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# STUDIES ON THE MILKY DISEASE OF JAPANESE BEETLE LARVAE

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## INTRODUCTION

In recent years, the milky disease of Japanese beetle larvae has become one of the most widely known of the biological control measures in the field of entomology, and its successful use by the Bureau of Entomology and Plant Quarantine of the United States Department of Agriculture and cooperating state agencies has been welcomed by an enthusiastic public.

The propagation and artificial dissemination of the causal organism of the disease have been conducted on a wide scale as a result of early work which demonstrated the feasibility of such methods in reducing the population of Japanese beetle grubs in tested areas. Studies on some of the fundamental biological aspects of the host-parasite relationship have been largely neglected. The work reported here is an effort to explain some of these relationships, particularly those concerned with the mode of infection, the pathogenicity of the disease organism, and the factors which affect the bacterium and its transmission from host to host. Many aspects of the disease have not been completely elucidated, and some of the problems discussed need further experimentation before definite conclusions may be drawn. It is hoped, however, that some questions will be raised which will serve to stimulate other investigations.

## BACKGROUND AND REVIEW OF LITERATURE

### Discovery

Smith and Hadley (36) reported that observations made in 1921 indicated the susceptibility of Japanese beetle larvae to certain diseases, presumably caused by bacteria, fungi, or protozoa. In 1935, Hawley and White (25) reported that diseased larvae were frequently found in the field and that, due to disease, mortality was sometimes very high among grubs kept in cold storage for experimental purposes. They divided the numerous maladies encountered into three groups: the black group and the white group, both caused by bacteria, and the fungus group. They concluded that the black group was the most prevalent. Later, Hadley (23) found the white group to be more abundant, being present at most of the places longest infested by the Japanese beetle, although absent in areas more recently infested. He stated that the diseases in the white group were due to two, or possibly three, similar but distinct organisms. White and Dutky (47) demonstrated that in this white group, two in particular were the most prevalent and were designated as Type A and Type B milky disease. The maladies were so named because the infected blood

of the larvae becomes chalky white in color due to the presence of great numbers of bacterial spores. Two different spore-forming bacteria were found to be the causal agents which Dutky (12) described and named *Bacillus popilliae* for Type A and *Bacillus lentimorbus* for Type B. In addition to establishing the etiology of these two diseases, Dutky discussed the effects of temperature on the development of the organisms and described attempts to culture the bacteria on artificial media. Although vegetative forms of *B. popilliae* were obtained, no culture media were found upon which the bacteria would sporulate. Since this work of Dutky, published studies have been limited to the Type A organism, *B. popilliae.*

**Development of Method for Use of *Bacillus popilliae* in the Field**

In the absence of any artificial culture media upon which the bacteria could be produced, the Bureau of Entomology and Plant Quarantine developed a process for obtaining the spores from the beetle grubs themselves and incorporating them in a dust mixture for storage and field distribution. This process was described by White and Dutky (47, 48) and in greater detail by Dutky (15). In brief, the process is as follows: Third instar grubs are inoculated with a known dose of spores (usually one million per grub) and incubated until the disease develops. The infected grubs are then ground up, the spore content is determined, and the mixture is used to impregnate talc. This material is then reground and dried. The final dust product is standardized to contain 100 million spores per gram of dust.

White and Dutky (47) reported that this spore dust as well as living inoculated grubs, spore suspensions and infectious soil could be used in establishing disease among grubs developing naturally in the field. Their experiments on various dosages and intervals of application demonstrated that dosages of about 25 million to 1,500 million spores per square foot, made continuously and in spots, resulted in successful establishment of the disease. These workers suggested that the spread of the disease is naturally accomplished by birds, insects, skunks, moles and mice. Langford, Vincent and Cory (26) have shown that the adult Japanese beetle, too, may be responsible for the spread of the disease.

As to the effect of introducing the bacterial spores into field populations of grubs, White (44) presented data showing that in one treated area, in West Chester, Pennsylvania, the grub population declined from 39.5 grubs per square foot on September 2, 1938 to 11.0 per square foot on June 12, 1939, while the incidence of disease increased from 4.0 per cent to 43.9 per cent. In a nearby untreated area, the population declined during the same period from 39.3 grubs per square foot to 13.6, while the disease increased from 7.6 per cent to 30.4 per cent. A check area, at a greater distance from the treated area, during the same time interval, showed a decline of from 45.5 to 26.5 grubs per square foot, but practically no disease was found. In these areas, the 1939 population was greatly reduced by drought, but

the disease persisted and was recovered as far as 500 yards from the original treated area. White further reported the persistence of the milky disease in field plots in spite of abnormally extreme weather conditions of cold, wetness and dryness. White and Dutky (47) stated that larval reductions of over 90 per cent had been demonstrated in certain field plots during a single season. One case cited was at Cape Charles, Virginia, where the larval population was reduced from an average of 121 to six healthy grubs per square foot during the period from July to September, 1939. (The number of diseased grubs present, if any, on the latter date was not mentioned.) In the corresponding check area, 74 healthy grubs per square foot were present at the end of that period. Two other surveys of treated areas were reported by White (45). One, at Springfield, New Jersey, showed a decline of from 40 grubs per square foot on September 1, 1939 to 5.4 per square foot on June 29, 1940, during which time the disease increased from 19 per cent to 60 per cent. The other survey, made at Perry Point, Maryland, showed a decline of from 37 grubs per square foot on August 24, 1939, to 6.3 per square foot on June 17, 1940, while the incidence of disease increased from 4 per cent to 67 per cent.

### Further Applications of the Method

The studies just mentioned constituted the preliminary work upon which was based the spore-dust distribution program of the Bureau of Entomology and Plant Quarantine and the cooperating agencies of those states most severely infested by the Japanese beetle. Most of the spore dust has been produced at the Moorestown, New Jersey, laboratory of the Bureau of Entomology and Plant Quarantine, the various states assisting to a greater or lesser extent by supplying grubs for inoculating and processing or by supplying diseased grubs for processing (White and Dutky, 48).

The manner of field distribution was based upon the conclusion of White and Dutky (48) that "if two grams of standardized spore dust is applied per spot.....at intervals of ten feet, where a moderate to heavy larval population occurs, the organism within three larval feeding seasons will be distributed throughout the untreated portions by local migration of the larvae". The general distribution program has been outlined by White and Dutky (48) and White and McCabe (49). A number of states have reported in more or less detail upon their share in the program. Among these are Connecticut (Garman, Schread, Brigham and Smith, 22; Garman, Brigham, Schread and Smith, 20, 21; Schread, 33), Delaware (Stearns, Fassig and Beacher, 40; Chada, Ditman and Daigh, 7; Rice, 32), Maryland (Cory, 8; Cory and Langford, 9), New York (Smith, 34; Smith and Daniel, 35; Wheeler, 41, 42; Buchholz, 5, 6; Wheeler and Adams, 43), Pennsylvania (Light, 27), Rhode Island (Eddy, 16), and Virginia (French, 19). Most of these reports describe the method of field treatment used in the respective states and summarize the locations treated and

amounts of inoculum used. The papers of Cory (8), French (19), Smith (34), and Wheeler and Adams (43) include a discussion of the Japanese beetle problem and the general nature of the milky disease as a control measure.

A limited amount of field experimentation has been done since the preliminary work discussed above, upon which the field distribution program was based. Wheeler (41, 42) treated field plots with spore dust in a dosage series for comparison with untreated plots. Although data were not given for each dosage, summaries were given for the populations present and the incidence of disease for three generations of beetle grubs: 1940-1941, 1941-1942 (Wheeler, 41) and 1942-1943 (Wheeler, 41, 42). A comparison of his data indicates that, for the three generations, the population of grubs, presumably at about the same time each year, was eight, ten, and 3.5 grubs per square foot in the untreated plots while it was five, seven, and 2.4 per square foot in the treated areas. The incidence of disease for the three generations was 0.5, 12 and 39 per cent, respectively, in the untreated plots and 21, 41 and 40 per cent in the treated plots. It was obvious that, by the third year, the disease in the untreated (but adjacent) plots was as extensive as in the treated plots. Within the one generation, 1942-1943, the population declined from 29.9 grubs per square foot to 3.5 grubs per square foot in the untreated plots, and from 20.6 to 2.4 in the treated plots (Wheeler, 42). Wheeler and Adams (43) reported further data for New York State, indicating significant differences in incidence of disease among grubs in treated and untreated areas.

Data presented by Garman, Brigham, Schread and Smith (20) for Connecticut, showing populations in three treated areas and four untreated areas for from one to three years, indicated no well-defined population trends, but a definite build-up of the disease was noted the third year. In 1943, Schread (33) observed a decreased incidence of disease in the fall as compared to that in the spring in spite of an increase in the fall grub population. He attributed this to less favorable soil temperatures encountered in the fall.

Hawley and Dobbins (24), in summary form, have credited the milky disease with being one of two major agencies responsible for declines in Japanese beetle populations over a period of years, the other agency being summer droughts.

The scope of the distribution program may be indicated by the following summary of the number of sites treated, acres treated, and pounds of spore dust used, quoted from the report of White and McCabe (49).

| | Number of sites treated | Number of acres treated | Pounds of spore dust used in treatments |
|---|---|---|---|
| Connecticut | 1,937 | 1,408 | 2,639 |
| Delaware | 3,963 | 2,071 | 4,694 |
| District of Columbia | 44 | 1,893 | 2,254 |
| Maryland | 35,140 | 21,989 | 44,796 |
| Massachusetts | 6 | 6 | 10.5 |
| New Jersey | 461 | 818 | 1,041.5 |
| New York | 649 | 2,106 | 3,046 |
| North Carolina | 10 | 310 | 697 |
| Ohio | 138 | 146 | 789 |
| Pennsylvania | 2,238 | 1,926 | 3,052 |
| Rhode Island | 27 | 40 | 84 |
| Virginia | 752 | 785 | 1,016 |
| West Virginia | 8 | 5 | 13 |
| Total | 45,373 | 33,503 | 64,132 |

These data summarize the colonization work done from 1939 through 1942. Distribution work done since 1942 has not been completely reported.

The magnitude of the distribution program may be appreciated if the data for Maryland alone, which has been treated more heavily than any other state, are considered. Langford (personal communication) has estimated the total amount of spore dust used in Maryland from the time the work was started through 1944 to be about 62,500 pounds. This represents a total of about 2,836,952,673,125,000 spores, which, if spread uniformly over the entire land area of Maryland, would provide each square foot with over 10,000 spores. But, in addition to this quantity, Maryland (Cory and Langford, 9) has also applied 13,925 pounds of inoculated soil, the spore content of which was not standardized.

In addition to the government sponsored distribution of spore dust, private individuals throughout the beetle area have treated local areas, purchasing commercially prepared dust. The method of spore dust preparation is protected by U. S. Letters Patent No. 2,258,319, issued to the Secretary of Agriculture, who may license individuals or concerns to produce and market this biological material. Several such licenses have been granted, and the spore dust is on the market under various trade names.

This is not the first time that such a biological material has been commercialized for the control of turf-feeding insects. In 1891, Marlatt (28) reported that a Paris firm, Fribourg & Hesse, produced the fungus *Botrytis tenella* (*Beauveria densa* Lk. Vuill) on artificial media "upon a vast scale". The spores were sold in tubes to agriculturalists for the control of white grubs. Trial tubes were advertised at 50 centimes, while the commercial package cost six francs. In use, the contents of the tubes were scattered over the bodies of white grubs contained in an earthenware vessel, the bottom of which was covered with earth. The grubs became diseased in six hours, when they were taken out of the container one by one and placed at a depth of about

20 centimetres in different parts of the field to be treated. These diseased grubs were supposed to serve as foci of infection for the natural grub population. Apparently, business in the sale of these fungus spores flourished for a while but the disease did not develop in the field as expected and, after a few years, nothing more was said about it.

There is no doubt that, in magnitude, the use of the milky disease organism will far exceed that of any other entomogenous bacterium or fungus, even including d'Herelle's bacillus. *Coccobacillus acridiorum*, and the chinch bug fungus, *Beauveria globulifera*, which were widely disseminated artificially during the latter years of the past century and the early years of the current century in attempts to control grasshoppers and chinch bugs in the Middle West. (Review by Smith, 37).

### Biological Studies

The work on the production of the milky disease bacteria and their dissemination in the field has largely taken precedence over studies on their fundamental biological relationships to the host insect. Dutky (14) has described techniques for handling the spores and grubs in testing the value of the disease and has reported (Dutky, 13) that a number of scarabaeid larvae other than the Japanese beetle are susceptible to the disease, among them being *Anomala orientalis*, *Autoserica castanea*, *Cyclocephala* (*Ochrosidia*) *borealis*, *Phyllophaga anxia*, *P. bipartita*, *P. ephilida*, *P. fusca*, *P. rugosa*, *Strigoderma arboricola* and *Strigodermella pygmaea*. He found *Macrodoctylus subspinosus* not susceptible, whereas conflicting statements were made regarding *Cotinis nitida*. White (46) considered the possibility of antagonism between milky disease and *Tiphia* parasites of Japanese beetle grubs, and found that the parasites could complete their development on hosts infected with milky disease and that the disease apparently has no effect on mortality of *Tiphia* larvae within the cocoon. He thus concluded that these two biological control agencies are compatible within the same area.

Beard (2) found that the incidence of milky disease bore a definite relationship to the dosage of spores whether injected into the body cavity or ingested by grubs feeding in contaminated soil. He also presented evidence which indicated some resistance to the disease under certain conditions.

## THE CAUSAL ORGANISM, *Bacillus popilliae* DUTKY

Dutky (12) has described *Bacillus popilliae*, and it is sufficient for the present discussion to point out that the vegetative form of the bacterium is a slender, nonmotile rod (Figure 1) and that the spore form has a very characteristic shape. The entire spore-structure is pyriform to spindle-shape (Figure 2, A), consisting of a sporangium containing an endospore and a refractile body. When stained by a

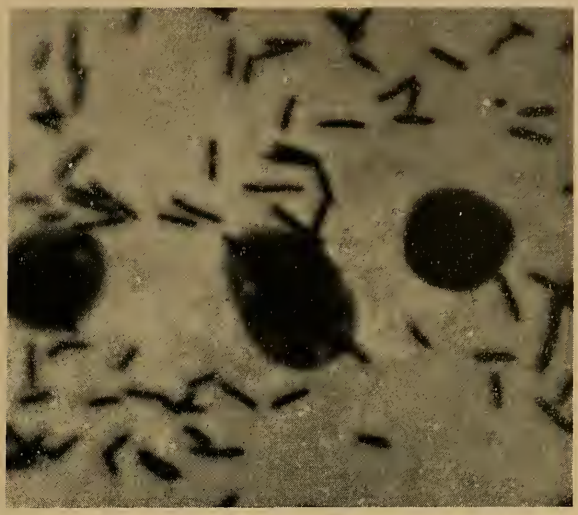

FIGURE 1. Photomicrograph of vegetative rods of *Bacillus popilliae*. (Large structures are blood cells of beetle grub.) X 1500.

carbol fuchsin spore stain, the endospore alone remains prominent (Figure 2, B). In unstained preparations, the external protoplasm is only faintly visible, and the prominent endospore and refractile body are so placed as to suggest a footprint in outline (as indicated by arrow in Figure 2, A). Because of this characteristic shape, the spores of *B. popilliae* may be distinguished from other bacteria or debris with reasonable certainty.

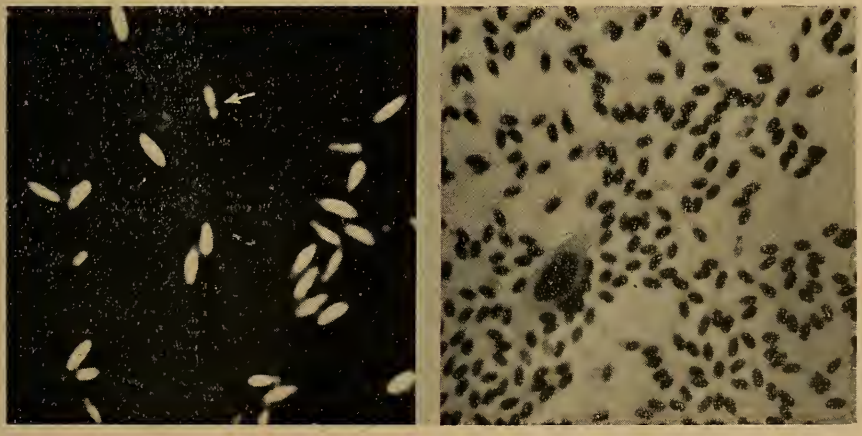

FIGURE 2. A. Nigrosin preparation of spores of *Bacillus popilliae*. (Arrow points to footprint shape characteristically seen in unstained smears.) X 1000.

B. Spores of *Bacillus popilliae* stained with carbol fuchsin. Large object is blood cell of beetle grub. X 1000.

## THE HOST, *Popillia japonica* NEWM.

The Japanese beetle and its larva are so well known that little need be said in regard to its description or habits. In brief, the life cycle of the beetle is as follows: The adult beetles emerge from the ground during the summer, beginning about June 20 in southern Connecticut, and are active for about eight weeks. The beetles mate and the females fly to turf areas, where they deposit eggs in the upper part of the soil. The young grubs which emerge then feed on the grass roots and in their development molt twice before the onset of cold weather, when the grubs go down a few inches in the soil for winter protection. In the spring, the grubs approach the surface and again feed on the plant roots. After feeding for several weeks, the grubs pupate, and shortly thereafter the adult beetles emerge. There are thus two feeding periods for the grubs—the developmental period in the late summer and fall and the period in the spring prior to metamorphosis. Both of these are important in connection with the milky disease and its development.

## MODE OF INFECTION OF *Bacillus popilliae*

### Invasion Route of the Bacterium

The nature of the milky disease is that of a bacteriemia, since the bacteria develop, probably exclusively, in the blood of the beetle larvae. The mode of infection, then, involves the transfer of the bacteria from the exterior into the body cavity of the grub. Dutky (12) pointed out that the disease can be artificially induced by hypodermic inoculation of the spores of *Bacillus popilliae*. This, of course, places the bacteria directly in the medium in which they normally develop. When the disease is acquired naturally, the bacteria must reach the blood indirectly, as the body cavity is bounded externally by the integument and its tracheal invaginations and internally by the alimentary tract and diverticula. This means that the bacteria must actually penetrate the integument (or trachea) or the wall of the alimentary tract (or its diverticula). (See Figure 3). Although no experimental proof has been given, it has generally been assumed that the bacteria in the spore stage are ingested by the grub as it feeds on

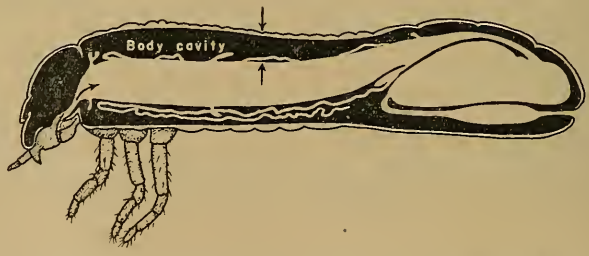


FIGURE 3. Diagrammatic longitudinal section of Japanese beetle grub, showing alimentary tract in white and body cavity in black. Arrows indicate routes of bacterial invasion to be considered.

grass roots or other plant material and that infection is thus via the alimentary tract.

A single experiment demonstrates that this assumption is correct. The mouths of a group of grubs were sealed with a synthetic resin, and the grubs were incubated for 17 days in soil containing sufficient bacterial spores to cause disease in approximately 60 per cent of untreated grubs. None of the grubs with mouths sealed contracted the disease, indicating that the normal invasion of the spores is not by penetration of the integument or tracheal walls. The fact that individual, isolated, grubs may become infected when exposed to contaminated soil is further evidence of bacterial invasion via the alimentary tract, as is the fact that the disease can be induced by oral injection of spores.

There remains, however, the possibility of entrance of the bacteria through open wounds such as might be caused by the nipping of other grubs when growing under very crowded conditions. This was thought by Hawley and White (25) to be the principal manner in which larval diseases in general were transmitted. This is contradicted by two tests. In one, two groups of grubs were incubated for 17 days at 78° F. in soil containing five concentrations of spores in a dosage series as previously described (Beard, 2). In one group, the grubs were wounded by snipping the integument with scissors. The grubs in the other group were left intact. Although mortality of the wounded grubs was too high to permit satisfactory analysis, the survivors showed an incidence of disease no greater than the intact grubs. The second experiment served to compare the incidence of disease among grubs reared under isolated conditions and under conditions allowing contact among the grubs. These two groups of grubs also were incubated for 17 days at 78° F. and in soil containing five concentrations of spores in a dosage series. In one group, the grubs were individually separated from each other by metal barriers and, in the other group, no such separations were provided. The quantity of soil provided in both groups was one cubic inch per grub (= population of 144 grubs per square foot if grubs are at one feeding level not exceeding one inch in depth). The incidence of disease resulting, as indicated in Figure 4, did not differ appreciably in the two groups. The slightly greater amount of disease among the grubs not isolated is not statistically significant, but it is possible for a secondary infection to occur among grubs thus reared. That is, when a healthy grub bites a diseased grub, thereby getting a mouthful of bacteria-containing blood, it may become infected. In one test in which healthy grubs were actually observed to bite diseased grubs and then incubated in sterilized soil, 54 per cent of the biting grubs became infected. Thus, when grubs are reared under conditions allowing contact with each other, there is the possibility that those grubs first becoming diseased may infect other grubs by being bitten.

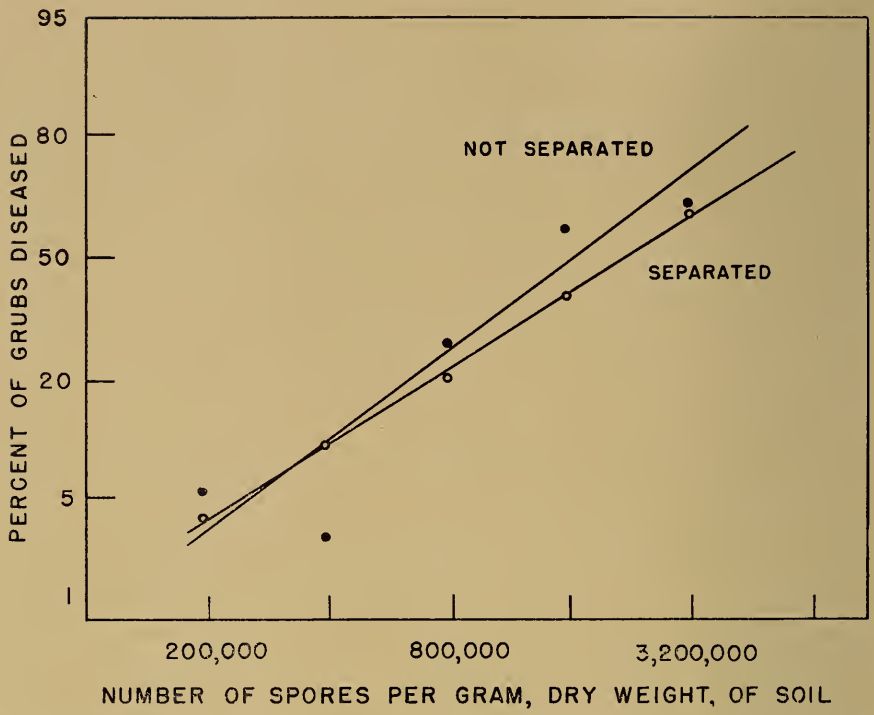


FIGURE 4. Incidence of disease among grubs incubated in isolation and among grubs not isolated.

## Infective Form of the Bacterium

There seems to be no doubt that the natural mode of infection is by way of the alimentary tract. The next question to be answered is, do the bacteria reach the blood from the gut as spores, or do they first germinate and enter the blood as vegetative forms?

One experiment demonstrates that the vegetative form may reach the blood. It was mentioned above that healthy grubs may become infected by biting diseased grubs. If healthy grubs are allowed to bite diseased grubs containing only vegetative forms of the bacterium, the disease may be induced in the healthy grubs. Because of the promptness with which the infection appears in the biting grubs, it is reasonably certain that the bacteria do not first sporulate in the gut and enter the blood in that form. It should be stated that not all grubs become infected after biting diseased larvae. Enough positive cases have been observed, however, to establish the fact that the disease may be transmitted by the vegetative bacteria.

Although this test indicates that the rods may reach the blood of the host, it does not prove that spores cannot reach the blood from the alimentary tract. Frequent observation of the blood of grubs

exposed to soil containing bacterial spores indicates that the first evidence of infection is the presence of vegetative forms, and not spores. Although this is by no means conclusive, it suggests that the spores germinate before reaching the body cavity. The following experiment serves to support this.

A group of grubs, known to be free from disease, was allowed to feed for 36 hours in soil containing a high concentration of bacterial spores. The grubs were then removed from the soil and placed individually on moist filter paper in Syracuse watch glasses. Half of the grubs were then incubated at a temperature of 78° F. to serve as controls. The other grubs were incubated at a temperature of 66° F. At this temperature the bacterial spores germinate very slowly, but the grubs are not inactive. It was believed that, if the spores were passively carried into the body cavity by the visceral activity of the grub, they could be recovered in the blood before they germinated. If, on the other hand, the entrance into the blood required the activity of the vegetative bacterium, assuming that the spore is itself inactive, no evidence of the disease would be seen within the time limits set up. Thirty-six hours later the grubs were examined. Of eight control grubs, five showed the presence of the disease, indicating a reasonably high rate of infection. Of nine grubs incubated at 66° F.. none showed the presence of bacteria in the samples of blood examined. A second examination made two days later indicated no change. Although a few spores present in the blood could easily escape detection when only a drop of blood from a grub is examined, the results of this test are in line with other observations, and it seems reasonable to conclude that the normal mode of infection is for the spores to germinate within the gut or its diverticula and the bacteria to reach the blood in the vegetative form.

### Localization of the Region of Penetration

The problem of localizing the point or areas where the bacteria penetrate the gut to reach the blood is a difficult one, and first involves a consideration of the morphology of the alimentary tract, which is illustrated in Figure 5.

The esophagus is relatively short, dilating to form a simple crop. The ventriculus is a straight tube from which arise three circles of caecal diverticula. The first circle, at the anterior end of the ventriculus, consists of short, blunt pouches, irregularly furcated. The caeca of the second circle are conical in shape, as are those of the third series. The latter caeca, however, are relatively short on the dorsal side of the ventriculus and become progressively longer toward the ventral side. These are directed anteriorly, usually more or less appressed to the gut. The hind gut is marked by three regions reflected upon each other in a Z-configuration. The anterior portion of the hind gut tapers posteriorly to its junction with the anteriorly directed, greatly enlarged, rectal sac. The third portion, the rectum proper, is directed posteriorly, where it opens to the exterior at the anus.

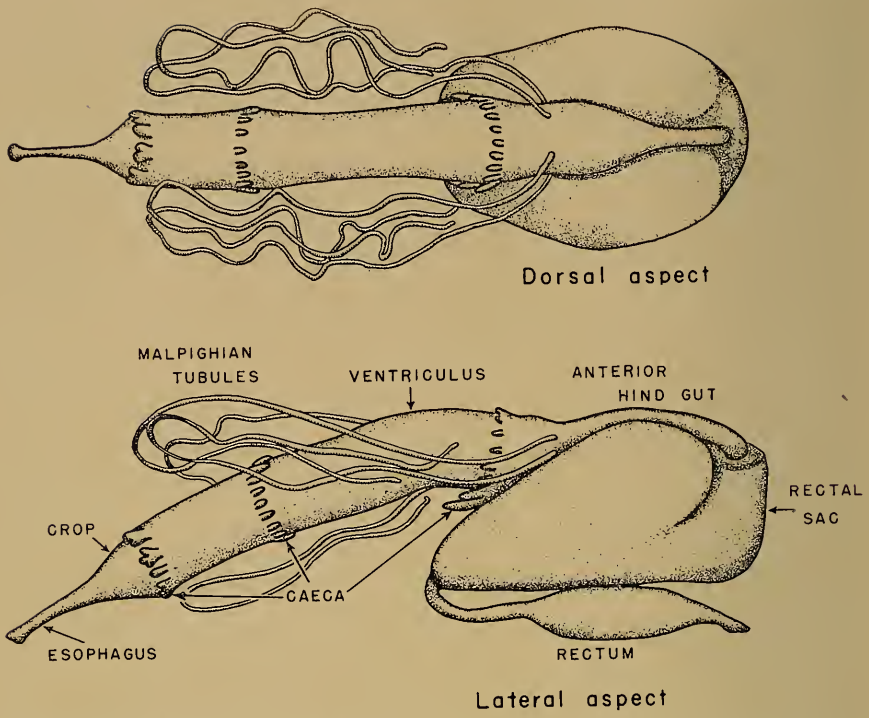


FIGURE 5. Diagram showing structure of alimentary tract of third instar Japanese beetle larva.

Two pairs of Malpighian tubules arise from the anteri or region of the hind gut, not far back of the third circle of gastric caeca. These tubules are long, looping anteriorly as far as the anterior region of the ventriculus.

The gut wall is certainly not a seive-like structure through which bacteria may pass at will. The gut and, in particular, the rectal sac, normally contains great numbers of bacteria and protozoa. At least some of these bacteria are very lethal when they reach the body cavity of the grub. This can readily be shown, for the grubs cannot tolerate any injury to the gut that permits the escape of the gut bacteria into the blood. Also, if these bacteria are cultured on nutrient agar and inoculated into the blood of healthy grubs, lethal effects usually result within 24 hours. Since these bacteria are far more virulent in the insect blood than the milky disease organism, and many of them are smaller in size, it follows that the mechanism of infection is one of selection, whereby *B. popillia* penetrates into the blood—but other bacteria are excluded. This is not without a counterpart in vertebrate pathology, for typhoid- and tuberculosis-causing organisms may penetrate the intestinal wall of humans, while other bacteria resident in the alimentary tract are unable to do so.

The presence of these naturally occurring bacteria in the gut makes the detection of the site of spore germination impossible by observational means. The spore, because of its characteristic shape, may be detected with reasonable certainty, but the vegetative rod cannot be satisfactorily distinguished from other rod-shaped bacteria commonly found in the gut. Experimental methods must then be used to localize the gut region where penetration of the bacteria occurs.

That there is such a restricted region of penetration seems evident from the low rate of infection that attends exposure of the grubs to contaminated soil. It has previously been shown (Beard, 2) that the rate of infection in a given length of time follows a probit response to the logarithm of the concentration of spores present in the soil in which grubs are incubated. This means that the chance of a grub becoming infected is a probability phenomenon. While the element of chance determines somewhat whether a grub actually ingests any bacteria or not, it is believed that if the bacteria could penetrate the gut at any point, the dosage-response curve would have a flatter slope, and a high rate of infection would not require such a heavy concentration of spores. Also, if the bacteria could penetrate at any point in the gut, enteral injections of bacterial spores should result in a very high rate of infection. This is not the case, as has been shown (Beard, 2). Extremely heavy doses of spores, even up to 300,000,000 spores per grub, injected enterally, fail to infect grubs with any degree of consistency. In many cases, the spores thus injected (orally) pass through the alimentary tract without germinating and, consequently, without infecting the grubs.

In one series of grubs injected orally with very heavy doses of bacterial spores, the anal opening was ligated by constricting the integument of the posterior segment with thread. Within two days, the spores were limited almost entirely to the rectal sac and rectum. A number of grubs living as long as seven to ten days after inoculation failed to show any bacteria in their blood and examination of the gut contents disclosed that large numbers of the spores were still present in the rectal sac and rectum. This would tend to eliminate these two posterior regions of the intestine as penetration routes of the bacteria into the blood.

Although oral injection of spores cannot be relied upon to infect Japanese beetle larvae with milky disease, some grubs may be infected in this manner. In about an equal proportion of cases the disease may be induced by enteral injections made anally. When injections are made in this way, the hypodermic needle is inserted into the lumen of the rectum as far anteriorly as the junction with the rectal sac. Large numbers of spores may thus be introduced into these posterior portions of the hind gut. Subsequent dissections of the gut indicate that the injected spores are not limited to these regions, however, but may be found in limited numbers, even in the ventriculus.

Since both oral and anal injections of spores may cause disease, the region of penetration must be reached by both, still assuming that there is a single, localized region where germination and penetration of bacteria occur. If the rectal sac and rectum are excluded as possibilities, the most probable site of penetration is somewhere in the posterior region of the ventriculus or the anterior region of the hind gut.

The problem may be approached in another way, by the use of the ligature method. Japanese beetle larvae do not tolerate ligation as well as some insect larvae. A large proportion of ligatured grubs die within a day, principally due to a rupture of the gut and a consequent liberation of bacteria, lethal when present in the blood. Under favorable conditions, a few survive two or three days, or even longer. When grubs are allowed to feed at favorable temperatures in soil heavily contaminated with milky disease spores, infected individuals may be detected in three days by blood examination. The disease organism seems to require this length of time to reach the blood after being ingested. Accordingly, grubs were allowed to feed for two days in soil containing a high concentration of spores. They were then removed from the soil and kept individually in watch glasses. Ligatures were tied around the grubs at various points. On the third day, the blood of the grubs was examined microscopically for disease. Dead grubs, grubs showing contaminating bacteria, and grubs showing no disease, were, of course, discarded. In the successful cases, note was made of the presence of the disease anterior or posterior to the ligature or both. The grubs were then dissected to determine the position of the ligature relative to the alimentary tract. The results noted are indicated in Table 1.

Table 1. Presence of Disease in Exposed, Ligatured, Grubs

| | Location of ligature | Presence of disease bacteria | |
|---|---|---|---|
| | | Anterior to ligature | Posterior to ligature |
| 1 | Behind Malpighian tubule base | + | — |
| 2 | " | + | — |
| 3 | " | + | — |
| 4 | " | + | — |
| 5 | " | + | — |
| 6 | " | + | — |
| 7 | " | + | — |
| 8 | " | + | — |
| 9 | " | + | — |
| 10 | " | + | + (doubtful) |
| 11 | At base of Malpighian tubule | + | — |
| 12 | Between Caeca III and Malpighian tubule | + | + |
| 13 | Through Caeca III | + | + |
| 14 | Between Caeca II and Caeca III | + | + |
| 15 | " | + | + |
| 16 | " | + | + |
| 17 | " | + | + |
| 18 | " | + | — |
| 19 | Between Caeca I and Caeca II | + | — |
| 20 | At Caeca I | — | + |
| 21 | " | — | + |

The results observed here are somewhat confusing except in one respect. This test confirms other observations that the bacteria do not germinate in and penetrate the rectal sac and rectum. For out of ten cases, all but one doubtful one showed no disease present posterior to the ligature when this was placed behind the base of the Malpighian tubules.

The caecal diverticula might seem to be very likely locations for the germination and penetration of spores, since in other insects, notably the Heteroptera, the caeca serve as the normal habitat of bacteria supposed to be of a symbiotic nature. Moreover, the caeca have much of their surface surrounded by the haemolymph. However, if the ligature tests can be relied upon, the caeca probably can be ruled out. If the third circle of caeca was the site of germination and penetration, the presence of disease posterior to the ligature in case 12 and that anterior in cases 14, 15, 16, 17 and 18 could not be accounted for. If the second circle of caeca was responsible, the presence of disease posterior to the ligature in cases 12, 13, 14, 15, 16 and 17, that anterior to the ligature in case 19, and the absence of the disease posterior to the ligature in case 19 are difficult to explain. No conclusions can be drawn from this experiment regarding the first circle of gastric caeca. Ligature experiments are difficult to make on this due to the proximity of the caeca to the head region.

If the wall of the alimentary tract proper is the site of germination and penetration, the ligature tests indicate that the entire region from the first circle of caecal diverticula to the base of the Malpighian tubules is favorable, which is contrary to the evidence discussed above.

It should be noted, however, from Figures 3 and 5 that the loops of the Malpighian tubules extend throughout this region. If the Malpighian tubules are considered to be the site of germination and penetration of the spores of *B. popilliae*, many of the difficulties can be explained, assuming that the bacteria entered the tubule prior to ligaturing and penetrating the wall after the ligature was placed. The tubules extend as far anteriorly as the first circle of caeca, where the ligature was placed in cases 20 and 21, with no disease resulting anterior to the ligature. The absence of disease posterior to the tubules in cases 1 to 9, inclusive, has already been noted. The absence of disease posterior to the ligature in cases 18 and 19 can be explained on the basis that bacteria were present in the anterior regions of the tubules but not the posterior. Moreover, the bases of the tubules are in a position that can be reached by spores injected both orally and anally. The fact that disease is more frequently acquired when contaminated soil particles are ingested than when enteral injections are made is possibly due to the fact that the fluid suspensions in the latter case sluice the spores on through the gut, whereas the more solid material passes more slowly by the tubule orifices, thereby giving the spores greater opportunity to enter the tubules.

If the Malpighian tubules do constitute the invasion route of the milky disease bacteria, it seems as though incipient cases of disease could be detected by examination of the tubules. Tubules in grubs allowed to feed for a few days in soil containing high concentrations of spores have been examined. In a very few cases, individual spores have been presumably detected when the tubules were macerated. This has occurred infrequently enough so that the possibility of contamination must be considered.

Histologically, the walls of both the gastric caeca and the ventriculus would seem to offer a much greater mechanical barrier to a non-motile bacterium than the wall of the Malpighian tubules (Figure 6). The former possess prominent columnar cells, with little intercellular space, within the basement membrane. The wall of the Malpighian tubule, on the other hand, consists of a single layer of large cuboidal cells with obvious intercellular spaces, and bounded by the limiting membrane.

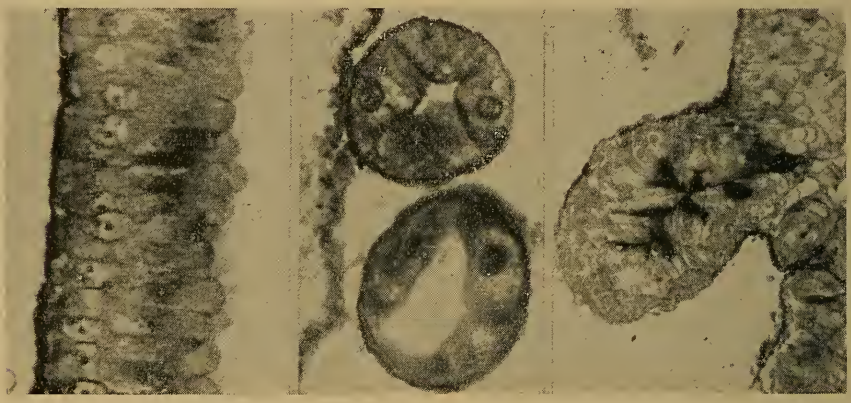

FIGURE 6. Photomicrograph of sections of ventriculus (left, X 380), Malpighian tubule (center, X 380), and gastric caecum (right, X 170).

One difficulty with the hypothesis that the penetration route is via the tubules is that bacteria ought to be able to go both ways through the wall. But no evidence has been observed to indicate that either the vegetative or spore forms of the bacteria are "excreted" via the tubules. In other words, why should a few bacteria be able to penetrate the tubule wall from the inside out, when large numbers of bacteria cannot penetrate from the outside in? It may be that the physiology of the germinating organism permits such penetration, while that of the dividing or sporulating organism does not. Or, the difference may lie in the tubule itself.

If the tubules from grubs with incipient infections could be transplanted into healthy grubs and cause disease, the hypothesis would be proved. A few attempts at this have resulted in the negative. Negative tests mean little, however, for the test may have been made at the

wrong time for the bacteria to be in the particular portions of the tubules transplanted.

Although the question remains an open one, in the light of experimental results observed to date, the invasion route of the bacteria through the Malpighian tubules seems more likely than through any other structure or region of the alimentary tract.

## THE COURSE OF THE DISEASE IN THE HOST

Although the cycle of development of *Bacillus popilliae* in the blood of the Japanese beetle larva has been described by Dutky (12), it is here reviewed and, for purposes of discussion, divided into definite phases.

### Developmental Phases of the Disease

Whether the disease is artificially induced by injection or naturally acquired, the first phase may be said to begin when vegetative rods of the bacterium first appear in the blood. This phase is termed the *invasion stage.* Of course, this must be preceded by the germination of the introduced spores but, since the ingestion or injection of spores does not necessarily insure a successful infection of an individual (Beard, 2), the appearance of the vegetative form initiates the bacteriemia. This is followed by a multiplication of the vegetative rods, which reach such numbers that the blood appears cloudy and assumes a somewhat granular appearance when viewed with the lower power of the microscope. This period of increase of vegetative rods is here termed the *incubation* phase. Just what induces sporulation of the bacteria is not known, but it occurs as a wave when the vegetative forms become exceedingly numerous. This *sporulation* phase continues until the definitive spore stage is reached by all those bacteria undergoing sporulation. At this time the bacterial cycle of development is considered to have reached *completion.* These developmental phases are illustrated diagrammatically in Figure 7.

The shadow cells mentioned by Dutky (12) were presumed to be rods incapable of sporulation.

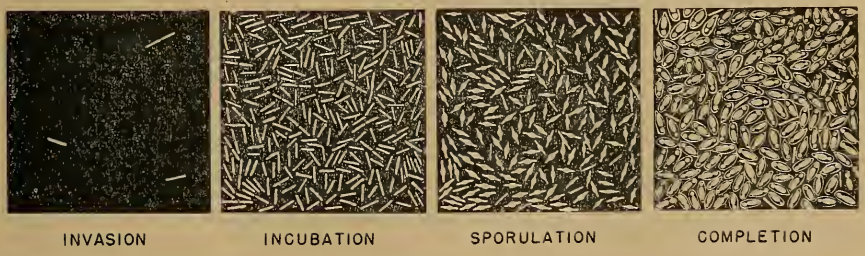


FIGURE 7. Developmental phases of *Bacillus popilliae.* Diagrammatic.

It is during the process of sporulation that the blood of the grub acquires its characteristic milky appearance. The blood of a grub with the disease in this phase appears distinctly granular when viewed under low power of the microscope (Figure 8), and the blood cells are largely obscured by the great numbers of bacteria.

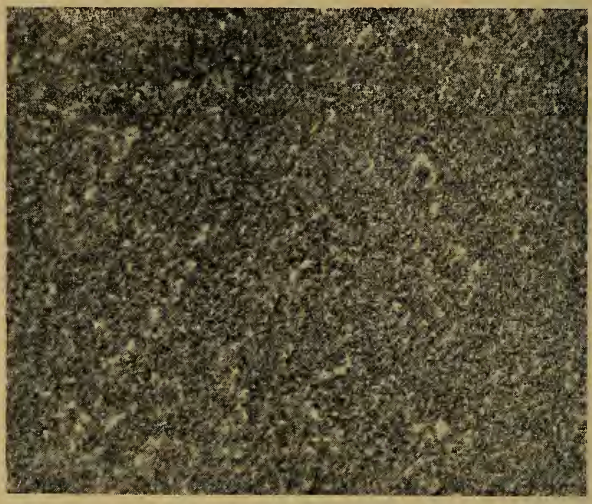

FIGURE 8. Granular appearance of diseased blood due to great numbers of bacteria, as seen under low power of the microscope.

Externally there may appear little difference between a healthy and a diseased grub (Figures 9 and 10). A diseased grub may be detected most easily by observation of the pericardial region and the posterior segments, where the opacity of the blood tends to cloud the dorsal blood vessel and the rectal sac. A greater opacity in the legs of the diseased grubs can be seen distinctly in the photographs in Figures 9 and 10. In determining disease by external observation, large fat accumulations may cause some confusion. However, if the posterior segments are constricted somewhat between the fingers, the fat body will be seen to move as a unit, whereas the opaque blood of a diseased grub can be seen to flow irregularly in the spaces between the hind gut and the integument.

In general, the opacity of the blood increases progressively until the grub becomes moribund, and a grub diseased for some time becomes more or less uniformly opaque (Figure 11). Dutky (12) stated that the number of spores in grubs reared at 30° C. (86° F.) reaches a maximum in 13 to 16 days after inoculation. If this is true, the subsequent increasing opacity must be due to the maturation of the spores rather than to an increase in the number of bacteria.

Whether or not the completed spores are able to germinate in the diseased grub and repeat a cycle is not known but, since intermediate

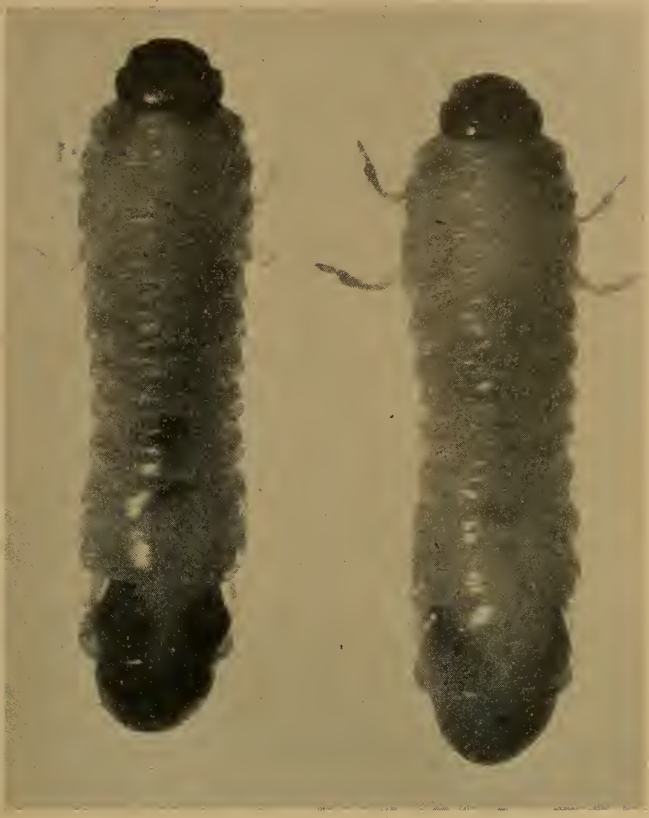

FIGURE 9. Dorsal view of healthy grub (left) and diseased grub (right). In latter, note greater cloudiness of posterior areas and greater opacity of legs. X 4.

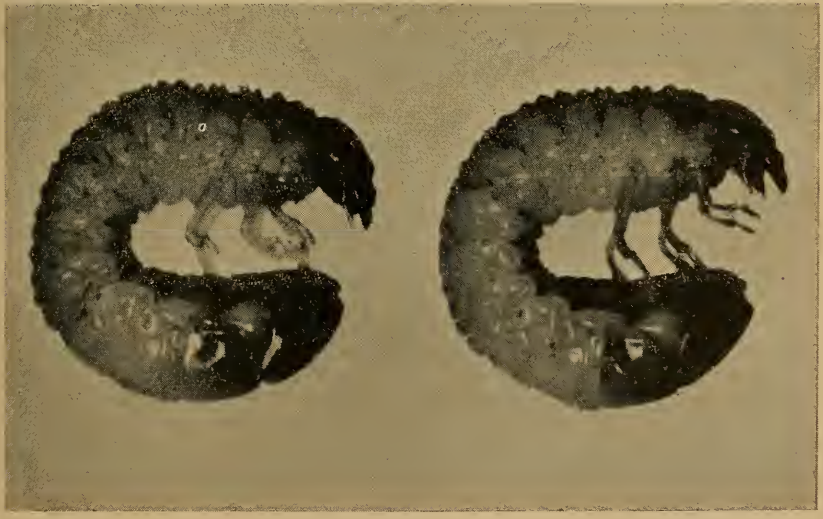

FIGURE 10. Lateral view of healthy grub (left) and diseased grub (right). Note greater opacity of diseased grub, particularly in legs. X 4.

sporulating forms are not in evidence after sporulation is once completed, it is doubtful if the number of bacteria present in a diseased grub is increased in this way. It has been observed in a few individuals that the development of the disease was arrested in the sporulating phase, the bacteria failing to reach the complete spore form. This was infrequent, however, and the cause is unknown.

### Time of Development of the Disease

The time required for the disease to develop is largely dependent upon the temperature. Dutky (12) observed a rectilinear relationship between the time of development, as judged by the appearance of macroscopic symptoms of the disease, and the temperatures between 62° F. (17° C.) and 93° F. (34° C.). It is very unusual, however, for microorganisms to grow at such rates as to give a rectilinear response with temperatures, and it may be that the criterion of macroscopic symptoms is not precise enough to judge the time of development. Certainly, in some grubs, the disease is macroscopically visible when the bacteria are in the last stages of the incubation phase, whereas, in others, it cannot be detected until sporulation is complete. When

FIGURE 11. Grub in advanced stage of disease showing uniform opacity all over body. X 6.

grubs were injected parenterally with spores of *B. popilliae*, incubated at temperatures of 86°, 76°, 70° and 66° F., and the development of the disease was observed by periodical microscopic examinations of the blood, the trends of development followed the pattern illustrated in Figure 12. It is obvious that at any given stage of development of the disease, the time required seems to follow more of an exponential than a rectilinear function of the temperature. This temperature re-

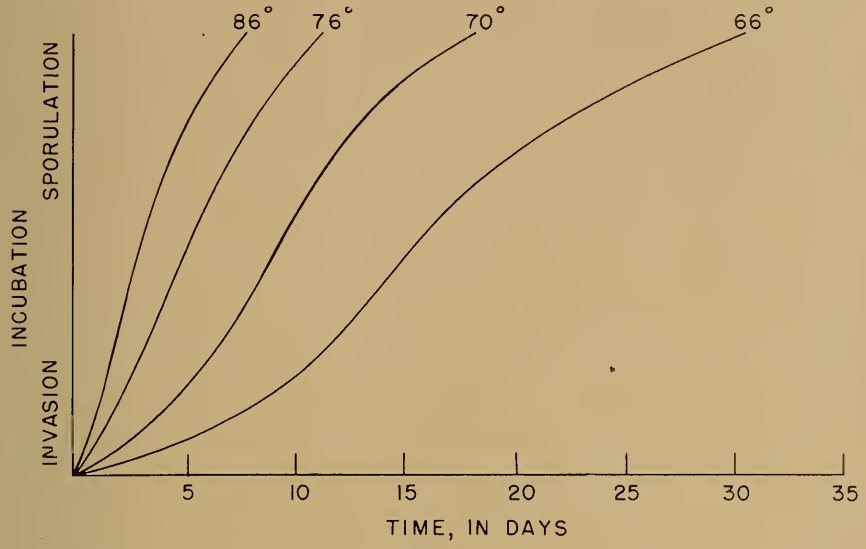


FIGURE 12. Time of development of *Bacillus popilliae* at different temperatures.

lationship has an important bearing on what may be expected from the field use of the bacteria in the control of the Japanese beetle larvae. It is obvious that when soil temperatures are lower than 70° F. the disease develops so slowly that a rapid turn-over and consequent build-up of the disease is impossible. Dutky (12) pointed out that approximately 16° C. (60.8° F.) is the temperature below which the disease will not develop. The beetle grubs, however, are not inactive at this temperature.

## PATHOGENICITY OF *Bacillus popilliae* IN BEETLE LARVAE

Paillot (31) has pointed out that, in contrast to microbial diseases of man in which the pathogenicity is principally due to secreted toxins, entomogenous bacterial diseases seem rather to cause a general suppression of the functions vital to the host. This general suppression may be more apparent than real due to the seemingly generalized physiological systems present in the insect organism. It is very likely that, if insect physiology were better known, and the action of bacteria upon insect hosts were more carefully studied, specific effects of

the invading organism would be discovered, whether toxins were involved or not. Sokoloff and Klotz (39), for example, have suggested that the pathogenicity of "Bacillus C" in the red scale of citrus may be due to toxic products arising from nitrate reduction in the host insect.

**Effect of the Disease on Larval Mortality**

In many respects, the relationship between the Japanese beetle larva and the milky disease organism is a favorable one for study. In contrast to many entomogenous septicemic (or bacteriemic) diseases which develop rapidly, killing the host in a matter of hours or very few days, the milky disease passes through a very methodical course of development, the time of which can be reasonably well controlled in the laboratory. Moreover, the infected grub does not necessarily die upon completion of the bacterial cycle of development, but may live for a longer or shorter time in the diseased condition. Grubs in the earlier instars succumb more promptly than the older grubs. For example, among the diseased first instar grubs observed in the laboratory, none lived more than 25 days after becoming diseased and, among diseased second instars grubs, none lived more than 55 days, whereas diseased third instar grubs have been observed to live over 70 days.

When death does come to the diseased grub, it certainly appears to be due to a general suppression of the vital functions, as Paillot stated. The exact point of death of an infected grub is difficult to determine and, in fact, may be a matter of definition. But, as the time for death approaches, the grub becomes less active and then moribund, losing first spontaneous movement and then response to tactile stimulation. At this time the circulation of the blood stops, and the bacterial spores settle down to the bottom of the body cavity. The white mass of settled spores can be seen easily through the integument of a dead diseased grub when it is turned over. Putrefaction does not seem to occur until the bacteria normally present in the alimentary tract gain access to the rest of the body. In fact, under dry conditions, there may be no putrefaction, the remains of the grub shrivelling to a small size.

One of the peculiar features of this disease is that pathologic symptoms are not coincident with the incubation, or even the sporulation, phase of the bacterial development, but are delayed until sporulation is to all appearances complete. It is generally believed that the spore form of a bacterium is inactive pathologically, and that any deleterious effect on the host can be traced to the metabolically active vegetative form. In this case, however, mortality among grubs in the early stages of the disease is not significantly greater than among healthy grubs, and grubs in the advanced stages of the disease may live for weeks without manifesting any signs of ill health or inhibited activity. This demonstrates that the pathogenicity of the milky disease organism is of a low order of magnitude and that, if any effects

are caused by the vegetative forms of the bacterium, their delayed manifestation is due to a residual condition or perhaps a cumulative action.

The mortality trends of diseased grubs can be considered by reference to the accompanying figure (Figure 13). This chart represents the result of a test in which third instar grubs were inoculated with milky disease spores and incubated at a temperature of 78° F. Grubs obviously diseased at the end of two weeks were then reared in groups at temperatures of 70°, 78° and 86° F., 64 grubs being used at each temperature. In other words, the grubs used were in the last phase (sporulation complete) of the disease. Similar groups of healthy

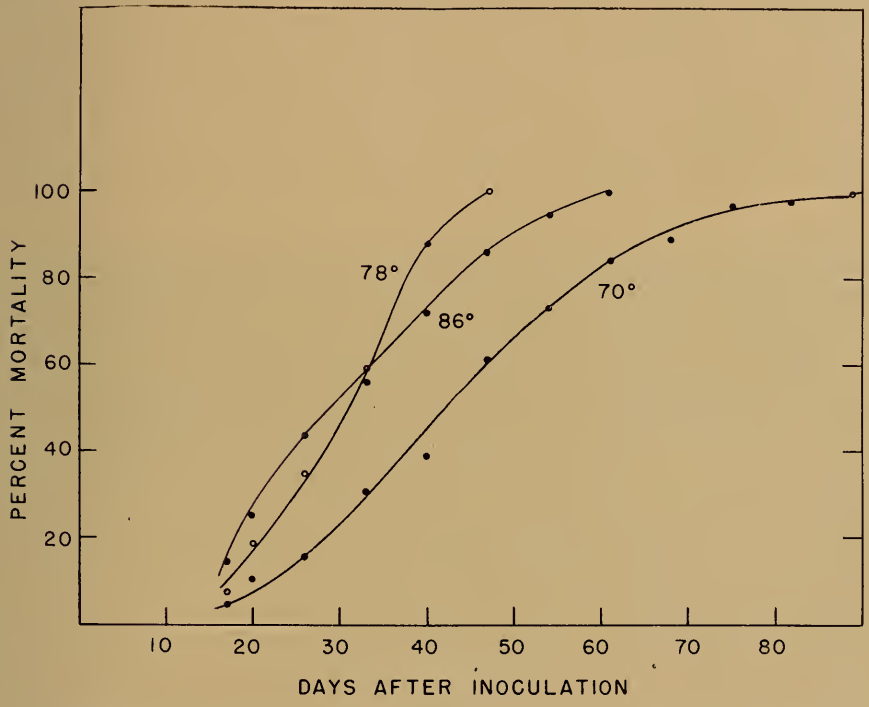


FIGURE 13. Cumulative mortality trends of third instar diseased grubs reared at 70°, 78° and 86° F.

grubs were reared under the same environmental conditions. Counts of the grubs made at intervals yielded data from which these curves were drawn. It is at once evident that grubs do not die at any definite time following the development of the diseased condition, but live varying lengths of time, presumably depending upon the vigor of the individual. It is seen that many individuals live for weeks or even months after their blood becomes loaded with bacterial spores. This is particularly true at low temperatures. In the field, it is not uncommonly observed that grubs becoming diseased late in the fall

will survive the winter, not dying until activity is resumed in the spring.

Not only do these diseased grubs remain alive for varying lengths of time, but they continue to feed until they become moribund. Evidence for this can be seen in the incubating compartments, where sprouted grass seed is provided as food for the grubs contained in cubic inch units of soil. Where grubs are absent or dead, the growth of grass is evident; where grubs are present, even though diseased, the growth of the grass has been inhibited. (See Figure 14.)

A comparison of the mortality trend of diseased larvae with that of grubs not diseased indicates that, for the most part, the diseased grubs do die somewhat sooner than the others. The accompanying chart (Figure 15) illustrates the mortality trends of diseased and non-diseased grubs reared at 78°. The results were fundamentally the same at the other two temperatures of 70° and 86°. At all three temperatures, however, a marked difference in the course of events appeared between the diseased and healthy grubs which limits the value of a comparison of these two trends. Until the time all diseased

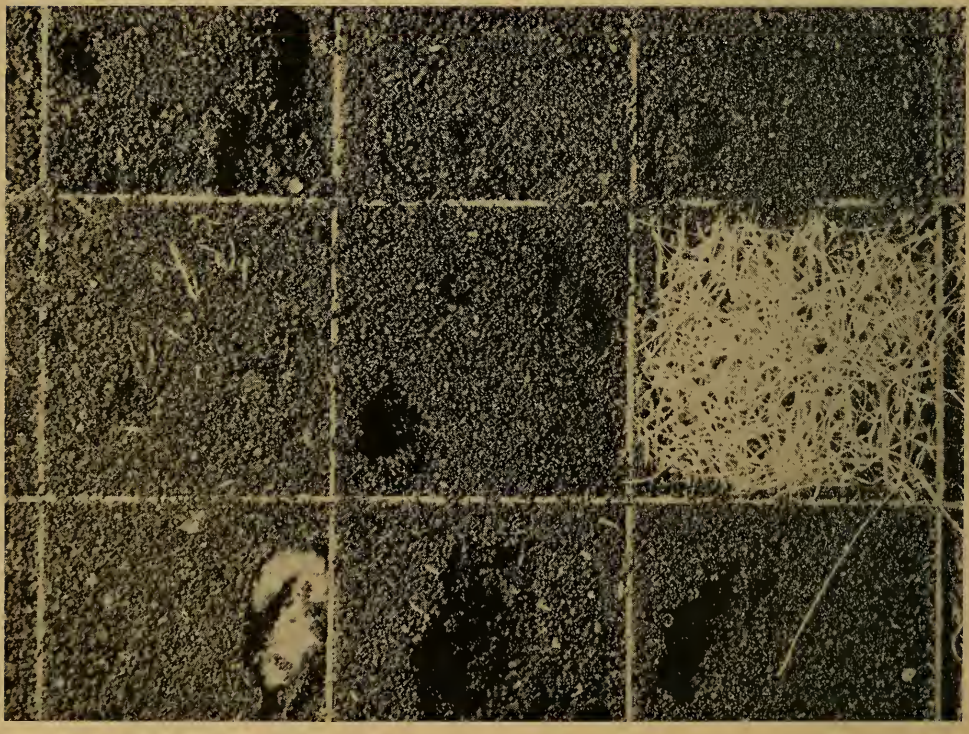

FIGURE 14. Surface view of incubation container. Evidence of grub feeding seen in inhibition of grass growth. Presence of grass indicates dead grub.

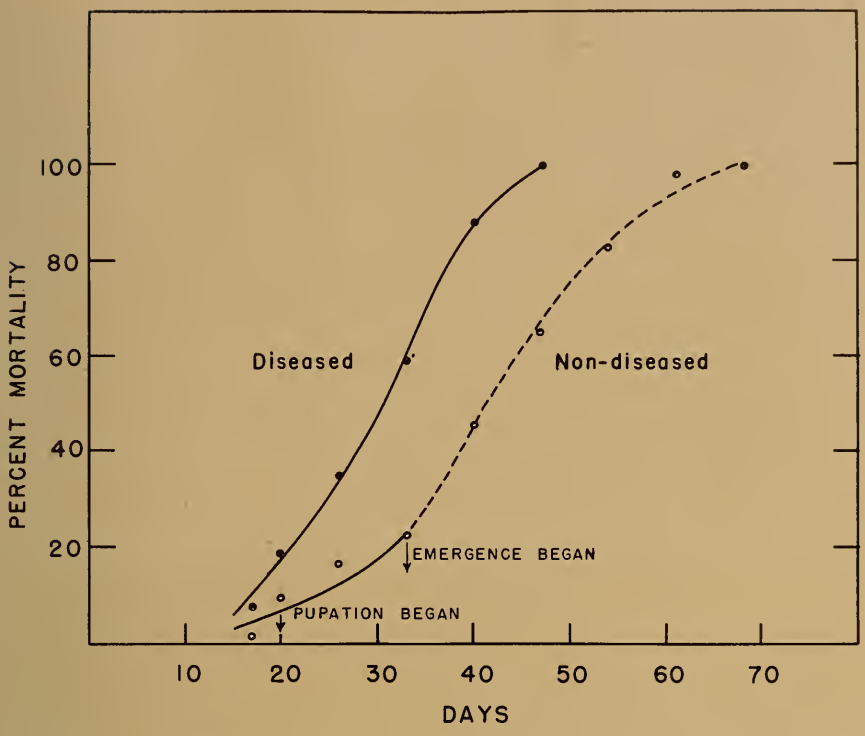


FIGURE 15. Cumulative mortality trends of third instar grubs reared at 78° F.

insects under observation died, in this case (78°), 47 days after inoculation, they remained in the larval stage. On the other hand, the healthy grubs began to pupate 20 days after the beginning of the experiment, and adults began to emerge two weeks later. Since the adult is really a different organism from the larvae, the rest of the curve (shown as a broken line) was calculated by disregarding those individuals becoming adults.

### Effect of the Disease on Molting and Metamorphosis

It becomes apparent that the disease does inhibit metamorphosis, for certainly many of the diseased grubs lived long enough to transform, if they were capable of so doing. This might at first glance seem opposed to the work of Langford and co-workers (Langford, et al., 26), who reported that diseased larvae are capable of metamorphosis. The explanation lies in the fact that a rather close relationship exists between the development of the disease and the ability of the host to metamorphose. If, at the normal time of pupation, grubs contain bacteria in the invasion, incubation, or even the sporulation stages, metamorphosis proceeds in an apparently normal manner. Grubs containing mature spores, on the other hand, are, with rare exceptions,

incapable of pupating, regardless of how long they may live and feed. This again emphasizes the delay of any effect caused by the vegetative form of the bacteria.

If metamorphosis is inhibited, it is possible that molting of the younger grubs might, too, be affected, and such is the case. Out of 40 diseased first instar grubs, only one molted to the second instar and, of 61 diseased second instar grubs, three molted to the third instar. The question at once arises as to whether this inhibition is merely due to the fact that the grubs were killed by the disease before they had a chance to molt—particularly when it is observed that the younger grubs do succumb earlier than the third instar grubs. Of course, it is impossible to know if any given individual grub lived long enough to molt, as the time of molting is indefinite and cannot be predicted. But, when groups are considered, the mortality trend of diseased grubs can be compared in respect to time with the molting trend of healthy grubs, as in the accompanying chart (Figure 16). It

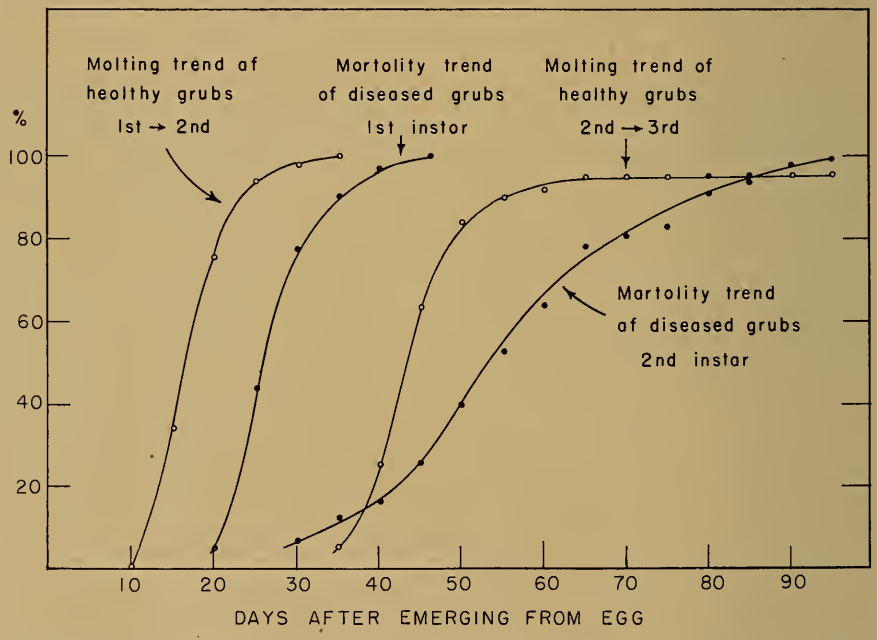


FIGURE 16. Comparisons of molting trends of healthy grubs with mortality trends of diseased grubs in early instars.

is obvious that, as a group, the diseased first instar grubs did indeed live long enough—and more—to molt, but were unable to do so. With the diseased second instar grubs and the second ecdysis, the picture is essentially the same, although it is clear that a few individuals, at least, did not live long enough to molt. It is possible for a grub to become infected in one instar and molt before the disease completed its

development but, with few exceptions, once the blood of an infected grub becomes milky (sporulation-completion stages of the disease), further development of the host is prevented.

From the standpoint of the individual grub, it may be concluded that the disease, while not immediately lethal, does shorten life expectancy, particularly among the young grubs, and it does inhibit molting and metamorphosis, provided the disease is fully developed.

**Physiological Effects of the Disease**

The physiological effects of the disease which might be responsible for these general effects may now be considered.

No necrosis of any organ system or tissue is evident and, prior to the moribund condition, no difference in activity is apparent between diseased and healthy grubs. The association of the bacterial spores with the fat body and wall of the alimentary tract as noted by Dutky (12) doubtless is incidental and without pathological significance. The blood, however, supporting the development of such large numbers of bacteria and, at the same time, fulfilling its vital functions in the grub must be considered in detail as to possible effects of the invading organism.

Due to the open circulation in the larval blood stream, the possibility of any mechanical block by the bacteria is remote, even considering the relatively large size of the bacterial spores, unless some form of agglutination sets in. This has never been observed. A simple, but striking, demonstration substantiates the absence of any mechanical interference with the circulation. If a diseased grub is cooled to the point where its circulation is retarded, the heavy spores settle to the lowest region in the grub. If then, the grub is brought to a higher temperature, the circulation is restored, and in a matter of minutes the spores again become dispersed uniformly throughout the body cavity.

The slow action of the bacteria in causing death of the host demonstrates the improbability of any lethal action by toxins secreted by the disease organism. This seems substantiated by the following injection experiment. Grubs, in groups of ten, were each injected with eight cubic millimeters of the fluids listed in Table 2. One group was uninjected for a control. The cumulative mortalities observed after three, six, nine and 12 days are tabulated. It is obvious that mortality was heavy only as a result of the inoculants containing spores. The fact that the spores, whether alive or dead, suspended in blood or in water, caused the heavy mortality implies that no toxins were involved, and that death was apparently caused by the mere introduction of a large quantity of foreign material. Diseased grubs, which do not die so promptly, contain far more bacterial spores than were introduced in the eight cubic millimeters injected, but the number is reached over a period of several days and the grub has a chance to accommodate itself to the invading organism.

Table 2. Mortality of Grubs Following Inoculation

| Inoculum | Cumulative mortality after | | | |
|---|---|---|---|---|
| | 3 days | 6 days | 9 days | 12 days |
| Uninjected | 1 | 1 | 1 | 2 |
| Distilled water | 2 | 2 | 2 | 2 |
| Heat-fixed blood[1] from healthy grubs | 0 | 0 | 0 | 0 |
| Heat-fixed blood from diseased grubs | 5 | 9 | 9 | 10 |
| Heat-fixed diseased blood minus spores[2] | 1 | 4 | 4 | 4 |
| Spores suspended in distilled water[3] | 5 | 7 | 9 | 10 |
| Spores suspended in distilled water[4] | 6 | 8 | 9 | 10 |
| Dead spores suspended in distilled water[5] | 8 | 8 | 8 | 8 |

[1] Blood is heat-fixed by immersing grubs in water heated to approximately 135° F. to prevent coagulation of the blood. (Yeager et al., 52).

[2] Supernatent fluid after centrifuging heat-fixed diseased blood.

[3] Spores washed in three changes of distilled water and suspended in water. Concentration per unit volume approximately the same as diseased blood (about 200,000,000 spores per grub).

[4] Same as above (3), but inoculum—approximately 126,250,000 spores per grub.

[5] Spores killed in water suspension by autoclaving. Inoculum per grub—approximately 170,500,000 spores.

A drop of blood drawn from the Japanese beetle grub quickly coagulates by a gelation of the plasma portion which entraps most of the blood cells. The coagulum can be gathered into a viscous drop with the point of a needle, leaving a small volume of more fluid "serum" containing scattered cells. Blood from healthy grubs was compared with milky blood, and no difference in the manner of coagulation appeared. Twenty-five samples of each were examined under the microscope, and the time required for coagulation was noted. Because the end-point, judged by gathering the coagulum into a distinct drop, was somewhat indefinite, the time was observed to the nearest five-second interval. No significant difference in coagulation time could be noted between the healthy blood and the diseased blood, the means being 18.8 seconds and 18.6 seconds, respectively, the range being from 10 seconds to 35 seconds in both cases. Whatever the chemical changes may be that are associated with coagulation, they do not seem to be affected by the presence of the disease.

Apparatus has not been available for testing the pH of the blood of individual grubs without exposure to air, but pooled samples of heat-fixed blood were tested with a glass electrode. Healthy blood showed a pH of 7.2, and the milky blood, 7.32. In another test, using centrifuged heat-fixed blood, the pH of healthy blood was 7.32 and of milky blood, 7.25. Apparently, these differences are not significant, for colorimetric, spot-plate determinations of pH in blood from individual grubs indicated as great a variation among healthy grubs as between healthy and diseased.

Although of questionable physiological importance, the specific gravity was found to be somewhat higher in diseased blood (1.034) than in healthy blood (1.027) as judged by one determination of pooled, heat-fixed blood samples. The higher value for milky blood is presumed to be due to the weight of the contained spores.

In view of the fact that amino acids account for the greatest component of the osmotic pressure in insect blood, and that the bacteria in the diseased grubs must utilize amino acids in their metabolism, the osmotic properties of healthy and diseased blood are of some interest. Attempts to determine the melting points of frozen, pooled blood samples failed to give satisfactory results. A simple osmometer indicated no significant difference between healthy and diseased blood.

Some of the inorganic constituents of diseased and healthy blood, heat-fixed and centrifuged, were determined by micro-chemical spot-plate methods.[1] These methods are not as precise as would be desired, but should show any major differences. The results of these tests are indicated in the following table:

Table 3. Inorganic Constituents of Blood of Japanese Beetle Larvae

| | Parts per million | |
|---|---|---|
| | Healthy blood | Diseased blood |
| Nitrate nitrogen | 1 | 1– |
| Nitrite nitrogen | 1 | 1 |
| Ammonia nitrogen | 1 | 2 |
| Phosphorus | 800 | 800 |
| Potassium | 500 | 400 |
| Calcium | 100– | 100– |
| Magnesium | 100 | 75 |
| Aluminum | 0.3 | 0.3 |
| Manganese | 1 | 1 |
| Sodium | 50 | 50 |
| Chloride | 1500 | 1500 |
| Sulfate sulfur | 500 | 500– |
| Copper | neg. | neg. |

It will be seen that, except for minor differences in the ammonia nitrogen, potassium and magnesium, the two samples were alike. It is doubtful if the small differences noted have any physiological significance.

Babers (1) found that, associated with the infection of larvae of the southern armyworm (*Prodenia eridania* Cram.) by *Bacillus cereus*, was a marked reduction in the number of cells present in the blood. No such reduction was noted in the case of Japanese beetle larvae infected with milky disease although, when the blood becomes milky, the cells become obscured by the exceedingly numerous bacterial spores. Blood cell counts were made from 20 non-diseased grubs and from 20 grubs infected with each stage of the disease—a total of 100. The mean number of blood cells for each group was found to be as follows:

[1] The writer is indebted to Miss Beverly Parker, of the Soils Department of the Experiment Station, for making these determinations.

Table 4. Number of Blood Cells in Healthy and Diseased Grubs

| | Number of cells per cubic millimeter of blood |
|---|---|
| Grubs not diseased | 26,290 ± 2655 |
| Grubs diseased | |
| Invasion stage | 22,508 ± 2094 |
| Incubation stage | 24,231 ± 2188 |
| Sporulation stage | 47,648 ± 2995 |
| Completion stage | 26,760 ± 2995 |

Except for those grubs in which the disease bacteria are sporulating, no significant differences in blood cell number appear among the groups of grubs. No definite reason for the marked increase at this one stage can be given. It is possible that some artifact in the counting technique is responsible, for the increase appears only at this one stage of the disease and shows no progressive change associated with disease development. On the other hand, it may be that the actual process of sporulation of the bacteria within the grub does modify the balance between the tissue fluids and the blood or in some other way affects the cell-plasma ratio of the blood. If this is true, the condition must be temporary, as the cell count of diseased blood in which sporulation is complete does not differ significantly from normal.

In two respects only, which are probably related, have differences appeared which might account for the over-all effects of the milky disease. For one thing, as is the case with other bacterial cultures, the oxidation-reduction potential of diseased blood is significantly lower than that of healthy blood. And, in the second place, if blood from a healthy grub is exposed to the air, it soon becomes very dark in color. Blood from a milky grub, on the other hand, usually fails to undergo this change (Figure 17). This melanization is funda-

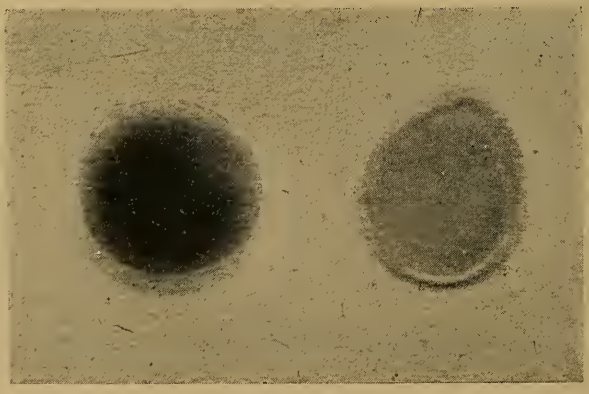

Figure 17. Drops of blood of Japanese beetle larvae exposed to air. Healthy blood, left; diseased blood, right.

mentally an oxidation phenomenon involving a chromogenic substrate and an enzyme such as tyrosinase or dopa-oxidase. That the disease interferes with one or more enzymes rather than with the chromogenic substrate is indicated by the failure of most diseased blood to blacken a solution of tyrosine, as does healthy blood. However, diseased blood does not consistently give negative results when tested with benzidine, gum guaiac and p-cresol, all of which give color reactions in the presence of oxidase. This variation is presumed to be due to the fact that grubs are affected to a greater or lesser degree. In fact, in a few grubs, the enzyme may not be affected. Also, it may be that phenolic oxidases, other than the one or more responsible for the melanization of blood, may be present and more or less affected by the disease. The bacterial spores themselves, washed in several changes of distilled water, failed to react to the tests for oxidases. Although the exact role of the disease in interfering with this enzyme system has as yet not been satisfactorily determined, it is believed that the disturbance of this or some similar enzyme system could account for the inhibition of molting and metamorphosis and a reduced longevity in the beetle grubs, without being immediately lethal.

There is no doubt that enzymes play an important part in molting and metamorphosis, and it is reasonable to assume that the oxidases in particular may be responsible in large measure for the utilization of metabolites in producing the increased energy required for molting, over and above that required for normal maintenance.[1] In the same way, an "energy debt" might account for reduced longevity, without being immediately lethal. It cannot be stated with finality that the destruction of the enzyme or enzymes responsible for the melanization of exposed blood also accounts for the inhibition of molting. It can only be stated that an oxidizing enzyme system is disturbed. Oxidizing enzymes are presumably required for molting. The milky disease inhibits molting and metamorphosis and shortens life expectancy in Japanese beetle larvae, possibly by destroying necessary enzymes. In this connection, it is of interest to recall that Dewitz (10) attributed the cause of metamorphosis to tyrosinase, which is most abundant at time of pupation.

The possibility of the milky disease destroying hormones necessary for molting and metamorphosis should be considered, but at present this seems unlikely. If the hormones in the Japanese beetle conform to the generalized pattern found in other insects (Bodenstein, 4), one would expect a hormone responsible for molting which also served to inhibit a different hormone responsible for metamorphosis. If only the molting hormone were affected by the disease, young infected grubs would be expected to metamorphose prematurely. If, on the other hand, only the metamorphosis hormone were affected, molting would not be inhibited. Consequently, if the disease does destroy necessary hormones, both the molting and metamorphosis hormones would have to be eliminated to produce the observed effects. Such

[1] Needham (29) and Needham (30) have reviewed the information on the biochemistry of metamorphosis.

hormones serve only a trigger action, and can function only if the organism is in a proper physiological condition. A disturbance of the latter would have the same end result as a destruction of the hormone.

Further work may clarify the picture and may demonstrate that other physiological processes are affected by the milky disease organism. The problems can be approached much more satisfactorily when a reliable and satisfactory artificial culture medium is found for the bacterium.

## SUSCEPTIBILITY OF JAPANESE BEETLE GRUBS TO MILKY DISEASE

### Effect of Spore Dose on Incidence of Disease

The susceptibility of third instar beetle grubs to milky disease has been discussed in some detail elsewhere (Beard, 2), but it should be mentioned here that the probability of a grub becoming infected increases with the spore dose, whether received by injection into the body cavity or by ingestion into the gut with food. This dosage response can be satisfactorily represented by a rectilinear curve when the data are plotted on a logarithmic-probability grid. In an unpublished thesis, Dutky (11) earlier noted a graded response when spore dosages ranging from 100 to 2,000,000 were injected parenterally. Although only ten grubs were inoculated with each dose, Dutky concluded that the expression (per cent infection) $1/2 = 3.1 \log_{10}N - 9.2$ roughly expressed the results, where N = the number of spores. Of his data, only three points could be plotted on a logarithmic-probability grid, but these could be very well represented by a straight line.

Although, in a given series of exposed grubs, the dosage-response is characteristic, it has been found that the level of response in different series may vary. This is because different groups of grubs may vary in their susceptibilities or different inoculants may vary in potency. The susceptibility of grubs and the potency of spores may act independently, antagonistically, or supplementally to give a higher or lower level of response. This dosage-response can be used to advantage in making comparisons for, by using a common lot of spores in a dosage series, the susceptibilities of different groups of grubs can be compared or, by using a common lot of grubs, the potencies of different lots of spores can be evaluated. Moreover, the dosage-response offers a means of bio-assaying the spore content of soil containing unknown numbers of *B. popilliae*. The dosage-response offers another advantage in serving as a check upon technique for very aberrant results may indicate previously unobserved natural disease or additional inoculum from unknown sources. Variations are, of course, to be expected and it should be pointed out that the variations are apt to be wider when the dosages are so low that the rate of infection is less than 15 per cent.

## Relative Susceptibilities of the Three Larval Instars

During the spring feeding period of the Japanese beetle grub, only third instar larvae are present in any number. During the feeding period in late summer and fall, all three instars are present and may be exposed to the disease.

The susceptibilities of the three instars were compared by exposure of the grubs to soil containing known concentrations of spores in a dosage series. The soil was prepared by adding measured quantities of spore dust, supplied by the Bureau of Entomology and Plant Quarantine, to soil which had been sterilized for at least two hours at 15 pounds pressure, and thoroughly mixed in a small, barrel-type mixer. The grubs in the first and second instars had been reared from eggs in the laboratory, in soil free from *B. popilliae.* The third instar grubs were field collected but, prior to testing, had been reared in sterilized soil for two weeks at a temperature of 78° F. in order that any grubs naturally diseased could be detected and discarded. Each group of grubs was incubated for 17 days at 86° F., after which the incidence of disease was determined by blood examination. Figure 18 graphically represents the results.

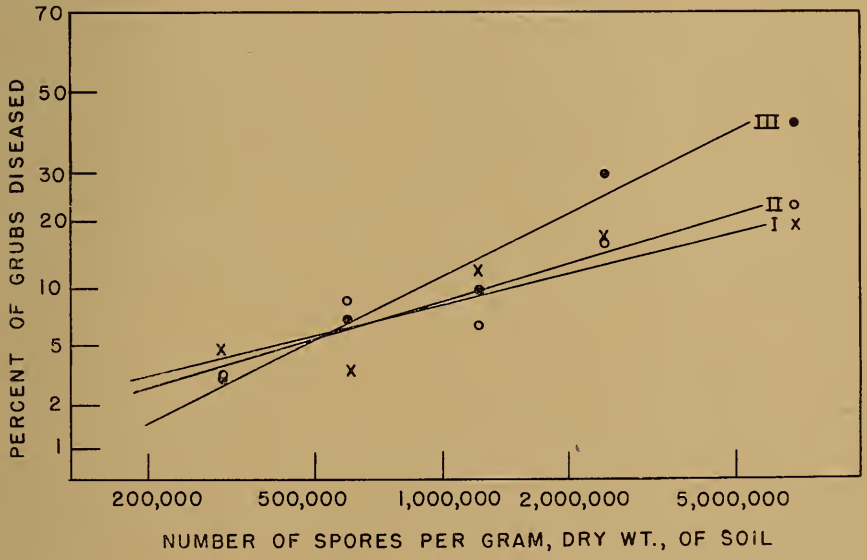


FIGURE 18. Incidence of disease among grubs of three instars incubated in soil containing milky disease spores.

It is obvious that the susceptibilities of grubs of the three instars are of the same order of magnitude when the grubs are reared for the same length of time at equivalent temperatures in soils containing the same inoculants. It should be pointed out, however, that the time interval used (17 days) was long enough for some of the grubs in the

early instars to molt. This meant that for a short period at and immediately preceding the molt, the grubs did not feed and, consequently, would not become exposed to spores. This might explain what appears to be a slight, progressive increase in susceptibilities among older grubs, although neither the slopes nor the positions of the regression lines show significant differences when analyzed statistically. The relative susceptibilities of the different instars will be given further consideration in a later section, in which the incidence of disease in a developing grub population is evaluated.

### The Factors of Chance and Resistance As They Affect Susceptibility

The susceptibility of the beetle grub to the invading organism involves a number of factors difficult to isolate. In the first place, the spores must be ingested with food and soil particles or with blood of a diseased grub. The probability of spore ingestion is increased with an increase in the feeding intensity and in the spore content of the soil. Once the spores are in the gut, the element of chance still determines whether or not viable spores reach the site favorable for germination and penetration into the body cavity. If all portions of the midgut were equally favorable for penetration, there would seem to be an infinite number of spots where germination could occur. If, as has been postulated, the Malpighian tubules constitute the invasion route, the spores must be caught at the orifice of the tubules in the hind gut. There is no reason to believe that the spores in any way actively seek the point of germination, but are presumed to be passively carried there. If they fail to reach such location, they pass on through and out the alimentary tract. To this point, then, except possibly for the feeding intensity, the likelihood of infection is largely due to chance and is independent of the physiology of the beetle grub.

The germination of the spore and the multiplication of the vegetative form, on the other hand, must be affected by the physiology of the grub. A physico-chemical environment favorable to bacterial development is presumed, but the presence of immunilogical or other antibiotic substances or the absence of essential growth factors may prevent the development of the disease. Phagocytosis of the bacteria has not been observed, but other defense mechanisms may be at work. Some evidence of resistance on the part of the beetle grub to the disease was presented by Beard (2). The nature of any such resistance is at present not understood, but it is doubtful if it is of an immunilogical nature in the usual sense. No grub has been observed to overcome the disease once it got started. Except in the rare cases previously noted in which the bacteria were arrested in the sporulating phase, the bacteria continue their cycle of development to completion.

### Effect of Preliminary Feeding by Grubs on Susceptibility to Disease

The factors that might affect the susceptibility of grubs have so far been inadequately investigated. A single test was conducted on the effect of preliminary feeding of grubs and their susceptibility.

Grubs collected in the field and held in cold storage were removed from storage, placed in a room where the temperature was maintained at 78° F., and allowed to feed on sprouting grass seed for six days. They were then inoculated with spores of *B. popilliae* in a dosage series.[1] Similar inoculations were made in grubs just removed from cold storage. The incidence of disease determined after two weeks of incubation is illustrated in Figure 19 and shows no essential difference in response between the grubs previously fed and those not. Neither the slopes nor the positions of the two regression lines differ significantly. It may have been that the preliminary feeding period was too short to

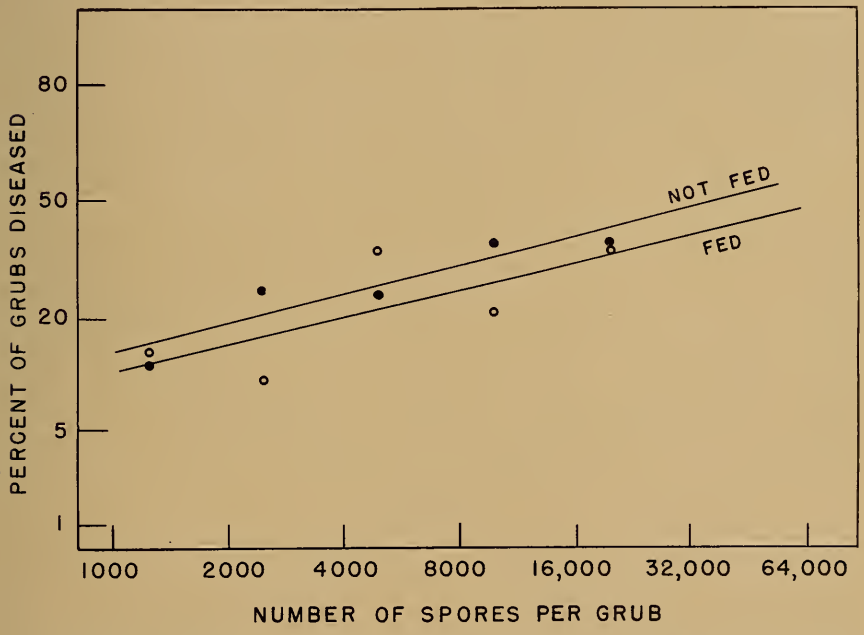


FIGURE 19. Incidence of disease among grubs fed for six days prior to inoculation and among grubs not so fed.

[1] In this and most tests reported below, 40 grubs were used at each dosage. Inoculations were made using five (or in a few cases, four) syringes. Ten grubs would be inoculated with one dose, syringes changed, and ten more grubs would be inoculated. The order of doses inoculated was chosen at random to achieve better randomization of the grubs injected. In early tests, the syringes were manually agitated prior to use. In later tests, a vertical, rotating (motor-driven) disc, in which the syringes were placed, served to agitate the suspensions and prevent the settling of spores. When the incidence of disease was evaluated, only survivors were examined. There is usually a small amount of mortality independent of the diseased condition, and it is impossible to tell, except in advanced stages, if a dead grub had become infected prior to its death.

modify the nutrition of the grub, but certainly under the conditions of this experiment, feeding had no effect on susceptibility. When, on the other hand, exposure to the disease is by ingestion of spores along with food, feeding intensity definitely affects the probability of ingesting doses sufficient to cause disease. But this is not a matter of susceptibility in the strict sense, however.

### Effect of Incubation Temperature on Susceptibility to Disease

In essentially the same way, the incidence of disease as affected by temperature was observed. Groups of grubs were inoculated parenterally with serial dosages of spores in the usual manner. Half of the grubs in each group were incubated at a temperature of 85° F. and the other grubs were incubated at 75° F. Due to the difference in rate of disease development at these temperatures, the incidence of disease was determined at several intervals rather than at any one definite time following injection. The total incidence of disease observed is illustrated in Figure 20. Again, as judged by this experiment involving only two temperatures, no statistically significant difference in incidence of disease appeared between the experimental groups.

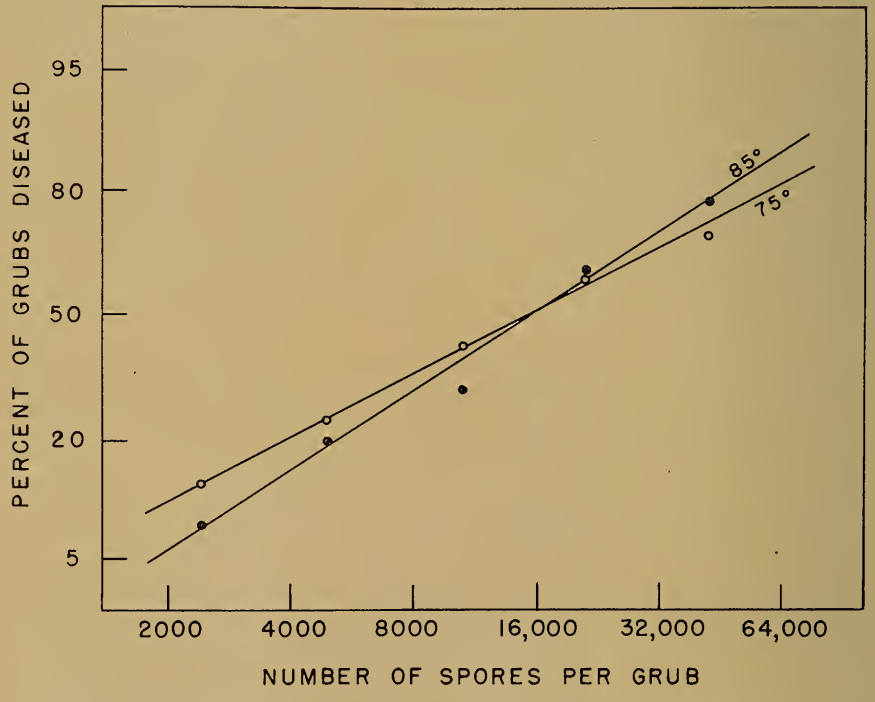


FIGURE 20. Incidence of disease among grubs incubated at different temperatures after inoculation.

## POTENCY OF SPORES OF *Bacillus popilliae*

### Effect of Age of Spores on Potency

Dutky (12) reported that spores in blood films dried for as long as 42 months gave a high rate of infection when injected into grubs. Although spores apparently remain viable for long periods when kept in this form, the possibility of a loss in potency must be considered. This was tested as follows: Spores in dried blood smears prepared from diseased grubs at different times were each inoculated in a dosage series into healthy grubs. Three of the smears used, prepared April 18, 1939, March 15, 1941, and December 16, 1942, were supplied through the courtesy of Dr. C. H. Hadley of the Bureau of Entomology and Plant Quarantine. A fourth smear, prepared February 16, 1944, was drawn from a diseased grub picked at random from cultures maintained in the writer's laboratory. Inoculations were made on February 24, 1944, and the incidence of disease was determined March 9, 1944. The ages of the spores were broadly considered to be one, 16, 35 and 58 months. The incidence of disease for the four series was found to be as represented in Figure 21. A remarkable uniformity of results is evident, from which it may be concluded that spores kept as dried smears not only may remain viable for extended periods of time, but do so with no loss of potency.

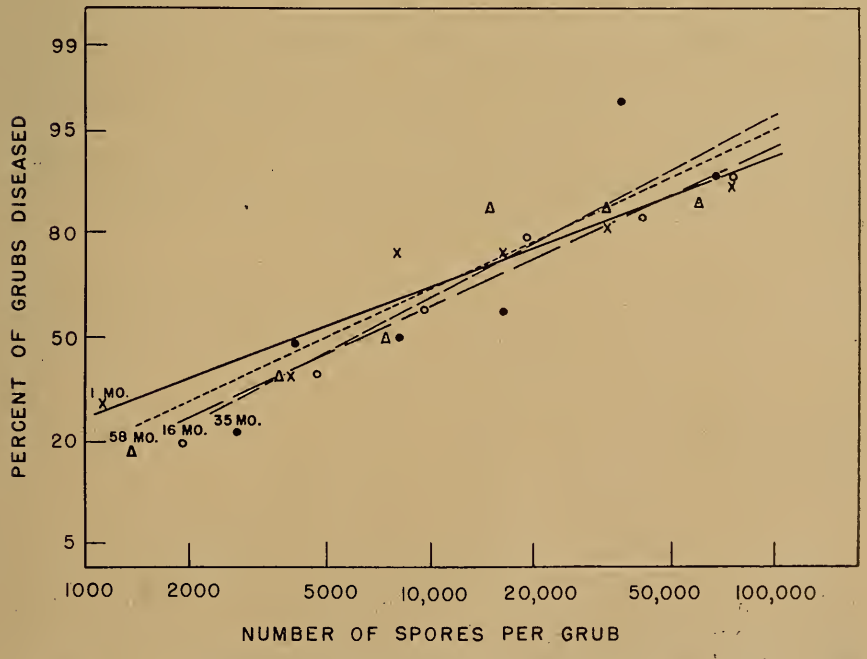


FIGURE 21. Incidence of disease among grubs injected with spores of different ages.

**Effect of Exposure of Spores in Soil to Weather**

It is of interest to know whether this resistance is still evident when the spores are incorporated in soil exposed to the weather. White (44) reported on the survival of the disease in the field under conditions of extreme weather, but no quantitative evaluation of the disease organism was made. One test was made with this question in mind.

On July 19, 1944, five kilograms of moist soil were thoroughly agitated in a small barrel-type mixer to assure a uniform sample. This sample was then divided into two equal portions by weight. To one sample were added 40 grams of milky disease spore dust supplied by the Bureau of Entomology and Plant Quarantine. An equal amount of spore dust was weighed, but placed in a jar for keeping in the laboratory. The soil sample containing the spore dust was then thoroughly mixed and placed in an eight-inch flower pot. The sample containing no spores was similarly potted, and equal amounts of grass seed were sown on the surface of each sample. These were kept indoors for a few days until the grass seed germinated, when they were taken outdoors and plunged in ground fully exposed to the sun. The summer was unusually dry, but the pots were artificially watered only a few times to keep the grass from dying completely. Towards the end of the experiment, heavy rains fell. After a total of 13 weeks, the pots were returned to the laboratory, and the contents were carefully sifted to remove all the soil from the grass. The two samples were individually mixed thoroughly, but to the one containing no spores was added the spore dust that had been previously weighed and kept in the laboratory. This, then, provided uniform soil samples with uniform inoculants except for the fact that, in one case, the spores were added before exposure to the weather and, in the other, the spores were added after exposure. Grubs known to be healthy were then incubated in the two soil samples for 17 days at 78° F. The incidence of disease was found to be as follows:

Table 5. Assay of Soil Containing Spores Exposed and Unexposed to Weather

| | Grubs present | Grubs diseased | Per cent diseased |
|---|---|---|---|
| Spores added to soil before exposure | 49 | 15 | 30.6 |
| Spores added to soil after exposure | 56 | 13 | 23.2 |

This experiment might have yielded more information if some kind of a dosage series had been used but it is at least evident in this case that exposure did not impair the potency of the inoculated soil. The apparent increase in potency is probably fortuitous.

**Relative Potencies of Fresh Spores and Spores in Dust Form**

In the test just mentioned and others involving inoculated soil, spore dust supplied by the Bureau of Entomology and Plant Quarantine was used as the source of spores rather than spores freshly drawn from diseased grubs. As it was desirable to check on the relative potencies of such soil mixtures, known numbers of fresh spores, in serial dilutions, were added to soil samples and thoroughly mixed. To similar soil samples were added weighed quantities of the spore dust, which is supposed to be standardized to 100,000,000 spores per gram. Grubs were incubated in the different soil samples for 17 days at 78° F., and the resulting incidence of disease determined. The results are indicated in Figure 22. Obviously, the spore dust was far less

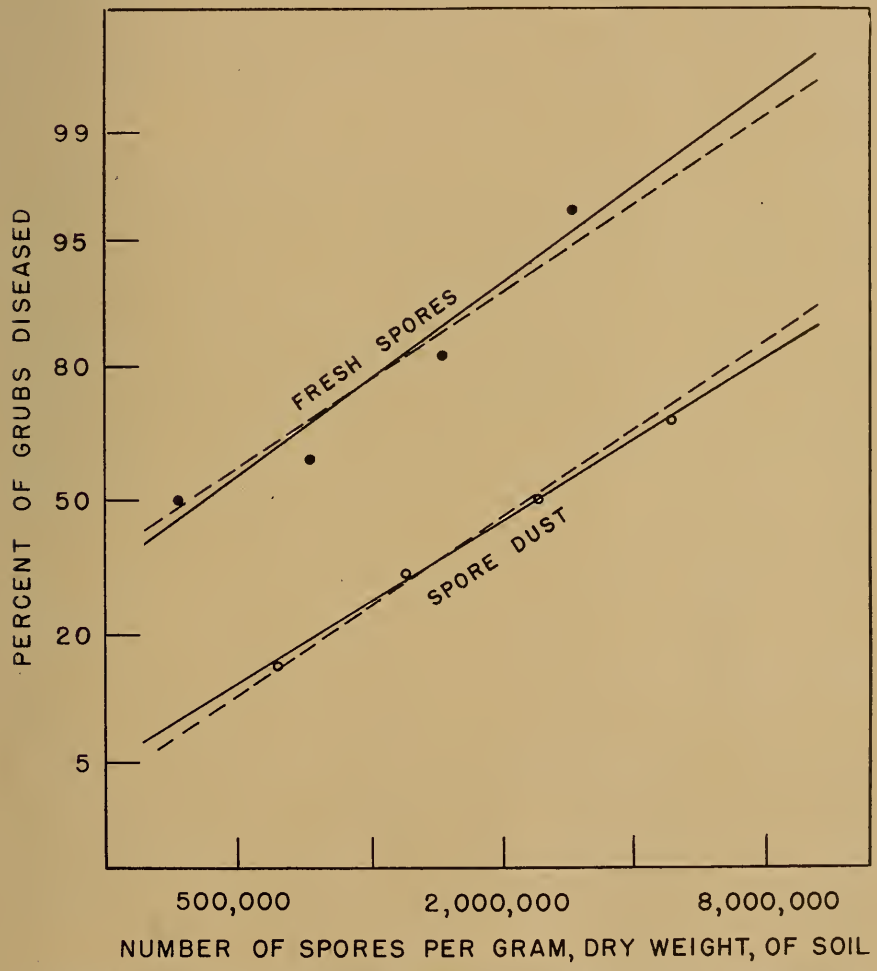


FIGURE 22. Incidence of disease among grubs incubated in soil containing spore dust and soil containing spores freshly drawn from diseased grubs. Solid lines—calculated regression; broken lines—regression based on combined slope.

potent than the spores freshly drawn from diseased grubs. Statistical analysis indicates that the two dosage-response curves can be represented by parallel lines, using a combined slope. If this is done, the potency of the fresh spores is found to be six times as great as the spore dust (i. e., six times as many spores in dust form are required to cause the same incidence of disease as spores freshly drawn from diseased grubs). The reason for this conspicuous difference is not known. The spore dust used was, to be sure, at least two years old, but in view of the experiment reported above, the age of spores, per se, does not seem to affect the potency. It may be that, by chance, either the spore dust used happened to be low in potency or the fresh spores used happened to be high in potency. Another possibility is that the actual method of processing the dust caused a loss in potency. In view of the wide-spread use of the spore dust, further comparisons should be made and, if consistent differences are found, the cause of the reduced potency should be found and, if possible, prevented.

### Effect of Ultraviolet Light on Potency of Spores

In general, ultraviolet light has an inhibitory effect on bacteria. If *B. popilliae* spores are thus affected, some loss in effectiveness might be expected when spore dust is applied to the surface of the ground and remains there for any length of time.

The effect of ultraviolet light on *B. popilliae* spores was tested in the laboratory. From pooled samples of diseased blood, smears were made on microscope slides. Covering the back of each slide was affixed a strip of tin-foil-lined red paper such as is usually wrapped around photographic film. These slides were arranged radially on a horizontally placed turntable mounted on the second-hand shaft of an electric clock. Suspended above the turntable at a distance of 12 inches was a G. E. type S-4 ultraviolet lamp. Although the intensity of ultraviolet emitted by the lamp is not known, the wave lengths emitted ranged from about 2,825 A. up through the range of visible light, including at least some red. Extraneous light was excluded from the set-up by means of black cloth. The turntable, revolving once per minute, assured uniform exposure, as it was known that the intensity of light differed around the source. Throughout the exposure, one slide was turned spore-side down to serve as a control. After eight, 24 and 72 hours, other slides were, in sequence, turned over. Thus, all smears were more or less equally exposed as to temperature, but the length of exposure to the light varied. After exposure, the spores in each smear were suspended in water, diluted serially, and injected into grubs. The grubs were then incubated for two weeks at 78° F., and the incidence of disease determined. The spores produced such a low incidence of disease that a dosage-response was not evident, and so could not be plotted. Consequently, the results are tabulated in Table 6.

The low amount of disease, even in the control, implies that the grubs were low in susceptibility or the spores were low in virulence.

Although an inhibitory effect of ultraviolet light is clearly indicated, the experiment was repeated in the same way except that the periods of exposure to the light were reduced to two, four and eight hours, and higher dosages were employed in the inoculations. Again, except among the grubs inoculated with the unexposed spores, the results

TABLE 6. INCIDENCE OF DISEASE RESULTING FROM INJECTION OF SPORES TREATED WITH ULTRAVIOLET LIGHT

| | Dosage | Per cent disease | | Dosage | Per cent disease |
|---|---|---|---|---|---|
| Control | 17,692 | 23 | 24 hour exposure | 20,769 | 0 |
| | 7,335 | 0 | | 5,212 | 0 |
| | 1,455 | 11 | | 2,364 | 0 |
| | 579 | 10 | | 1,916 | 0 |
| | 191 | 0 | | 579 | 5 |
| 8 hour exposure | 20,577 | 0 | 72 hour exposure | 8,462 | 0 |
| | 7,280 | 0 | | 4,054 | 0 |
| | 4,826 | 0 | | 2,364 | 0 |
| | 3,091 | 0 | | 766 | 0 |
| | 386 | 0 | | 579 | 0 |

were too erratic to plot in terms of logarithms and probits. This is not unusual when the response is of a low order of magnitude. They are tabulated in Table 7.

TABLE 7. INCIDENCE OF DISEASE RESULTING FROM INJECTION OF SPORES TREATED WITH ULTRAVIOLET LIGHT

| | Dosage | Per cent disease | | Dosage | Per cent disease |
|---|---|---|---|---|---|
| Control | 74,903 | 73 | 4 hour exposure | 73,938 | 0 |
| | 40,083 | 76 | | 34,818 | 0 |
| | 27,636 | 36 | | 19,783 | 3 |
| | 9,363 | 41 | | 9,242 | 24 |
| | 4,646 | 27 | | 4,586 | 0 |
| 2 hour exposure | 68,340 | 0 | 8 hour exposure | 73,166 | 0 |
| | 32,182 | 17 | | 34,455 | 0 |
| | 18,285 | 4 | | 19,576 | 3 |
| | 8,542 | 3 | | 9,146 | 0 |
| | 4,238 | 0 | | 4,538 | 14 |

Again, it is clearly evident that ultraviolet light has an inhibitory effect upon spores of *B. popilliae*. There is not much difference in results observed among the three lengths of exposure, so from this experiment the inhibitory effect of ultraviolet light upon milky disease spores can not be evaluated in terms of length of exposure. Undoubtedly, some of the spores received protection from the light by overlying spores. So far, no attempt has been made to correlate the effectiveness of the sunlamp in reducing spore potency with that of the sun itself. It is probable, however, that the spores are sufficiently

protected by the carrier in the dust and become incorporated in the soil soon enough so that, in most cases, the ultraviolet light is less important than other factors in limiting the effectiveness of the milky disease as a control agent against the Japanese beetle larvae.

### Effect of pH on Potency of Spores

If the potency of spores of *B. popilliae* was modified by differences in pH, the reaction of the soil to which the bacteria were applied might conceivably affect the results in terms of control. A pilot test was made by preparing a series of buffered solutions, ranging from pH 3.2 to 8.0. Into a 5 cc. portion of each solution was added a loopful of a concentrated suspension of spores. After 48 hours, uniform volumes of each solution were injected into ten beetle grubs. These were then incubated for ten days at 86° F., and the incidence of disease was determined. Since one or two grubs died in each of several groups, the results are expressed in the following table in terms of per cent of the survivors, although this represents a considerable extension of the data.

Table 8. Incidence of Disease Resulting from Injection of Spores Suspended in Solutions Differing in pH

| pH of solution | Per cent of surviving grubs diseased |
|---|---|
| 3.2 | 10 |
| 3.8 | 33 |
| 4.4 | 67 |
| 5.0 | 70 |
| 5.6 | 60 |
| 6.2 | 90 |
| 6.8 | 100 |
| 7.4 | 100 |
| 8.0 | 100 |

Although only ten grubs were inoculated with each suspension and the dose of spores was not standardized, the decrease in potency with lower pH seems too systematic to be fortuitous. There seems little doubt, therefore, that an acid environment is detrimental to spores of *B. popilliae*. Soils usually encountered in turf harboring Japanese beetle grubs do not have a range as wide as that employed above. Few soils in Connecticut, for example, exceed the limits of pH 4.5 and 6.5. Consequently, striking differences might not appear between spore-containing soils of different pH in regard to potency in causing disease. Nevertheless, the following experiment was conducted in an effort to determine any effect of soil pH on potency of spores.

Various soils naturally differing in pH have the disadvantage of varying also in elemental substances, texture, colloids, moisture equivalents, etc. Consequently, a soil of low pH was found and divided into six portions. By titration, an estimate was made of the amount of hydrated lime required to bring the soil up to the desired pH. The

lime additions were made, resulting in soils of six pH levels: 4.85, 5.00, 5.20, 5.49, 6.00 and 6.60. The soil of each pH level was thoroughly mixed and divided into portions each weighing 440 grams, moist weight. It was desired to have the soil contain bacterial spores for various lengths of time and then to test the samples for potency in causing milky disease. This could have been done in two ways, either by inoculating the soil samples at one time and testing at different intervals, or by inoculating the soil samples at different times and testing all at once. The latter method was employed as it was believed that spores from different sources might vary less than grubs coming from different sources or kept under different conditions.

Four soil samples at each pH were inoculated with serial doses of spores on each of three dates to give exposure intervals of two, four and eight weeks. Upon the addition of the inoculum, the sample was thoroughly mixed in a small crank-operated food mixer, and then placed in an ice-cream container. All samples were kept in a constant temperature room, and the moisture of the soil was maintained by the addition from time to time of uniform quantities of water. Pooled blood freshly drawn from a large number of diseased grubs served as the source of spores. The soil was insufficient for three complete series, so, for the soil of pH 6.6, two weeks exposure was omitted.

At the time of testing, 25 grubs were exposed to each soil sample, to which grass seed was added to provide food, for 17 days at 78° F. The incidence of disease was determined by microscopic examination of the blood.

The results obtained indicated that the three lots of spores varied considerably in potency, the lot exposed to the soil for four weeks being the most potent, and that exposed for two weeks being the least potent. The 17 different dosage series were calculated in terms of logarithms and probits, using weights and corrected probits (Bliss, 3). In only three series was a significant departure from linearity indicated by the chi-square test. In view of the generally consistent graded response to various spore doses, however, the straight line probably represents the best estimate of the response even in these three cases.

The spores exposed to the soil for four weeks produced the most satisfactory response, presumably because of their greater potency. The calculated regression lines for the series in this group are shown in Figure 23. To avoid confusion, the individual points are not plotted, but the chi-square test indicated that all series could be satisfactorily represented by straight lines. As determined by analysis of variance, it may be concluded that neither the slopes nor the positions of the regression lines differ significantly and, consequently, the effect of soil pH on spore potency may probably be considered negligible. The same conclusion, as judged by analysis of variance, can be drawn from the assay of soils containing spores exposed for two weeks and eight weeks. Even though the differences are not statistically significant, a suggestion can be seen from the data to indicate

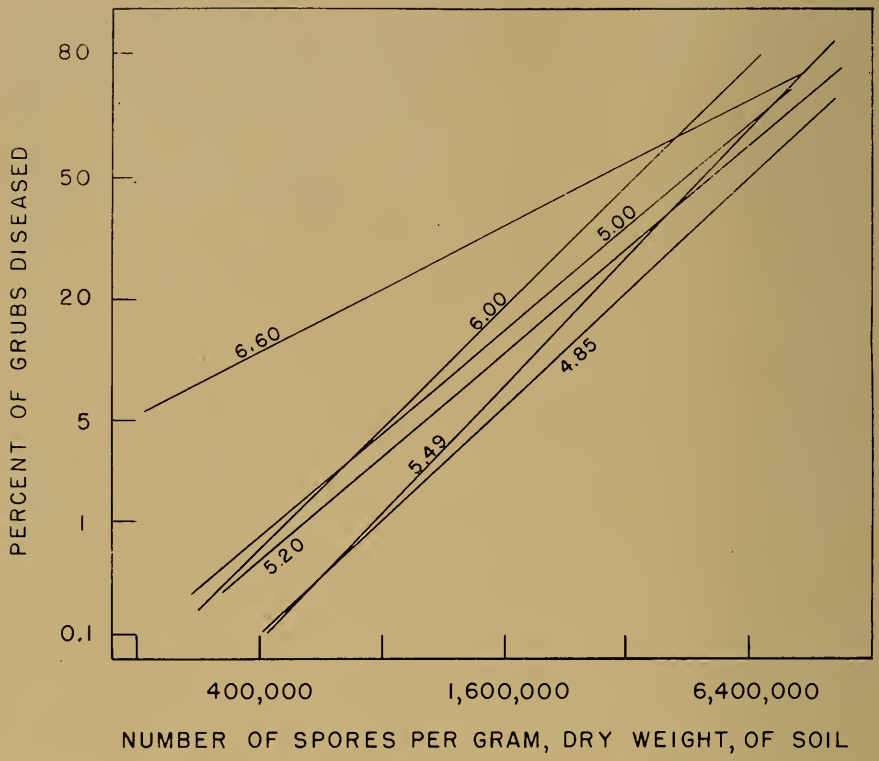


FIGURE 23. Incidence of disease among grubs incubated in spore-containing soil of different pH.

that the potency of spores might be favored by the higher pH. Table 9 indicates the number of spores required to cause disease in 50 per cent of grubs incubated in the soil, as calculated from regression lines using the combined slope of all series within each group. In two of the three groups, there seems to be some tendency for higher potency in soils of higher pH.

TABLE 9. NUMBER OF SPORES REQUIRED TO CAUSE DISEASE IN 50 PER CENT OF GRUBS REARED IN TEST SOIL

| Soil pH | Spores exposed in soil | | |
|---|---|---|---|
| | 8 weeks | 4 weeks | 2 weeks |
| 4.85 | 11,430,000 | 7,269,000 | 117,900,000 |
| 5.00 | 8,200,000 | 4,724,000 | 55,840,000 |
| 5.20 | 13,490,000 | 5,471,000 | 89,956,000 |
| 5.49 | 6,804,000 | 5,910,000 | 58,610,000 |
| 6.00 | 7,607,000 | 3,525,000 | 245,900,000 |
| 6.60 | 7,700,000 | 3,075,000 | |

From a practical point of view, the effect of soil pH on the potency of spores of *Bacillus popilliae* can presumably be neglected.

### Effect of Temperature on Spore Potency

The spores of *B. popilliae* are so resistant to heat that this is not a limiting factor in the field use of the bacteria. For laboratory practice, however, it is useful to know the thermal tolerance of the spores. Dutky (12) found that spores withstood the temperature of 80° C. (176° F.) for ten minutes. The present writer found that spore suspensions lost little potency when maintained at 194° F. for ten minutes, but that they lost almost, but not entirely, all potency when refluxed (212° F.) for ten minutes. An intermediate effect was noted with spores held at 203° F. for ten minutes. Since the loss of potency is probably affected by the length of exposure to high temperature, a test was designed to evaluate this. Since the greatest loss in potency occurs between the temperatures of 194° F. and 212° F., 205° F. was arbitrarily chosen for testing as it could easily be maintained in the water bath employed. Four series of spore suspensions in serial dilutions were prepared. For a control, one series was left untreated. The others, in test tubes, were placed in a hot water bath. A thermometer inserted in one tube served as a temperature check. When this reached 205° F., the temperature was maintained there. At the end of two minutes, one series was removed from the bath; at the end of six minutes, another series was removed, and the third was removed after 18 minutes. The suspensions were then used to inoculate grubs, which were reared for two weeks at 78° F. As with the ultraviolet treatments, the heat treated spores produced such low and erratic results that the incidence of disease could not be satisfactorily represented on a logarithmic-probability grid. Accordingly, the results are here tabulated. Although it is clearly evident that the potency of spores is largely lost at this temperature, the lengths of exposure, within the limits of this experiment, are of little difference in their effect. Undoubtedly, many spores were killed by heat before the temperature of the suspension actually reached 205° F.

Table 10. Effect of Heat on Potency of Spores

| | Per cent of grubs diseased | | | |
|---|---|---|---|---|
| | | Spores exposed to 205° F. | | |
| Dosage | Control | 2 minutes | 6 minutes | 18 minutes |
| 39,100 | 90 | 0 | 0 | 30 |
| 20,000 | 69 | 18 | 0 | 3 |
| 10,000 | 58 | 0 | 17 | 0 |
| 5,000 | 33 | 18 | 0 | 0 |
| 2,700 | 23 | 9 | 0 | 13 |

**Effect of Successive Passages Through Hosts on Spore Potency**

It is usually postulated that the virulence of pathogenic bacteria increases upon successive passages through a series of susceptible hosts of the same species. Some evidence of this has been observed with the milky disease organism, but when attempts have been made to evaluate critically such an effect, serious discrepancies appeared. For example, eight successive passages were made. In the first, second, fourth and eighth, inoculations were made in a dosage series for comparison. Inoculations in intermediate passages were made to continue the strain, but without regard to dosage and response. The number of spores required to cause 50 per cent disease among the grubs in the four observed series was estimated in round numbers to be as follows:

| | |
|---|---|
| First passage | 8,000 spores |
| Second passage | 1,000 |
| Fourth passage | 2,000 |
| Eighth passage | 70,000 |

Although the second and fourth passages indicate spores of greater virulence than those first inoculated, the spores used in the eighth passage seemed to be very low in virulence. It must be remembered, however, that the susceptibility of grubs, as well as the virulence of the spores is involved and, in many cases, cannot be distinguished. In such a test, when the successive transfers are made over a period of time, it is impossible to maintain a host population in a uniform condition. Also, the selection of spores from among the diseased grubs may have a bearing on the results. In view of these considerations, the problem may be principally one of technique.

Another approach was made which, in addition to throwing light on the problem of increasing virulence with successive passages, indicated a means of selecting a more virulent strain of the bacterium, if such were possible. It can be seen from the dosage response that very low doses cause disease in very few grubs. It seems reasonable to suppose that the few grubs which do become diseased when few spores are injected parenterally are more susceptible to the disease than other grubs, *or* the few spores causing disease in these grubs are more virulent than the other spores. Accordingly, spores were inoculated into Japanese beetle grubs in serial dosages of relatively low concentration. The grubs becoming diseased as a result of the lowest dose injected served as the spore source for the next series of inoculations. This was repeated for a total of five passages. Unfortunately, the supply of grubs available became depleted, and the experiment could not be properly concluded. The results obtained are represented in Figure 24. To avoid confusion, the mathematically fitted lines are shown without the individual points plotted. In each case, however, the regression was satisfactory, as shown by the chi-square test, and consistent with the numerous other responses to injection of spores in serial concentrations.

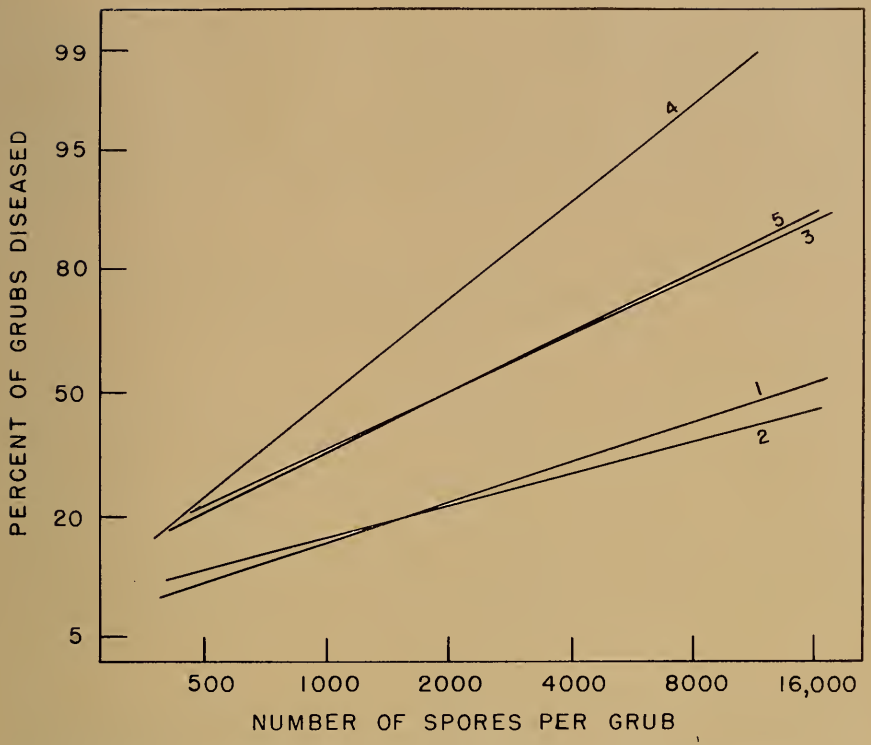


FIGURE 24. Incidence of disease among grubs inoculated with spores derived from successive passages through host. First to fifth passage, inclusive.

The results obtained from the first two passages are not significantly different. The third and fourth passages, on the other hand, show progressive and pronounced increases in the incidence of disease resulting from a given dosage of spores. The fifth passage fails to continue the trend, closely conforming to the third passage. This casts doubt on any generalization as to the selection of a more virulent strain of bacterium. Had an adequate supply of grubs been available, the selected spores could have been compared with spores obtained at random from other infected grubs to determine if the higher incidence of disease was due to increased virulence of spores or to increased susceptibility of grubs.

A repetition of this experiment had to be discontinued before it had progressed very far. Again, the reason was an inadequate supply of grubs due to an unusually high rate of mortality among those held in storage.

### Effect of Refrigeration of Spores in Water Suspension on Potency

In one experiment already reported (Beard, 2), it was very evident that spores lost considerable potency when suspended in water

and kept in a refrigerator for two weeks. This observation has not been consistent but, in one test in which spores in a water suspension were refrigerated for 20, 40 and 80 days, a progressive loss of potency was noted. In these cases, the effects of refrigeration and of contact with water were not segregated, and may, in fact, have little practical significance, as soil assays have indicated that spores may remain viable with little loss of potency when left over winter in the surface layers of soil outdoors.

## NUMBER OF BACTERIAL SPORES PRODUCED IN JAPANESE BEETLE GRUBS

### Number of Spores Produced per Host

Where interest centers around the production of spores of *B. popilliae* for artificial dissemination, the number of bacteria to develop within an individual host is of some importance. Dutky (12) stated that as many as 20 billion spores were found in a single individual grub. Langford, et al. (26) reported that the average number of spores produced in an individual was 500 million in the adult beetle and two billion in the larva. The maximum number in any one individual observed by the present writer was slightly over four billion, the average being, as Langford found, about two billion.

### Effect of Host Size on Spore Yield

The number of spores produced seems to bear little relation to the size of the host, as judged by weight. Twenty-three diseased grubs, chosen at random, were individually weighed in a weighing bottle, and the total number of spores present was determined by grinding the specimen in a mortar, suspending the material in a known volume of water, and counting the bacterial spores by the use of a blood-counting chamber. The number of spores produced, ranging from 650,000,000 to 3,462,500,000, showed a poor correlation with the body weight of the grubs, as can be seen from Figure 25.

### Effects of Incubation Temperatures and Inoculum on Spore Yield

It has been recommended that, for the production of spores, inoculated grubs should be incubated at the relatively high temperatures of 85 to 87° F. to yield the maximum number of spores. In this laboratory, however, mortality of grubs was enough greater at these temperatures, than when they were maintained at temperatures of 75 to 78°, to warrant a check on the effect of temperature and spore yield. At the same time, it was desirable to define the effect of inoculating dose upon spore yield, a correlation of which was noted by White and Dutky (47).

In an initial test, a series of grubs was inoculated at five dosage levels and maintained at each of three temperatures: 60°, 75°, and 85° F. No disease developed among the grubs held at 60° but, at the

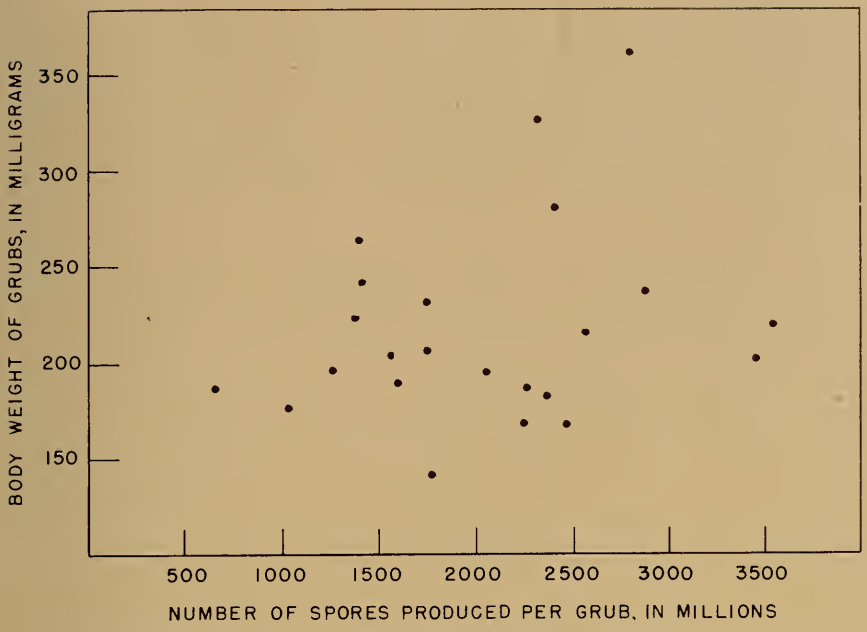


FIGURE 25. Spore yield relative to body weight of host grubs.

other temperatures, the characteristic dosage-response was obtained. Among the grubs held at 85°, those obviously diseased after 12 days and, among those held at 75°, those diseased after 14 days were dehydrated, weighed, and the spore yield determined in the same manner as described above. The results are tabulated below, the number of spores representing the mean of ten counts in each case and expressed as the number of spores per grub and also the number per gram of dry weight of grub:

TABLE 11. SPORE YIELD RELATIVE TO REARING TEMPERATURE AND INOCULUM

| Dose | Temperature | Number of spores per grub | Number of spores per gram of dry weight of grub |
|---|---|---|---|
| 41,954 | 85° | 74,000,000 | 1,317,567,567 |
| 21,139 | | 72,888,888 | 1,043,921,069 |
| 10,561 | | 209,600,000 | 3,475,953,565 |
| 4,977 | | 150,983,333 | 2,629,608,127 |
| 2,384 | | none diseased | |
| 41,954 | 75° | 33,636,363 | 906,418,422 |
| 21,139 | | 95,507,692 | 1,640,808,770 |
| 10,561 | | 115,500,000 | 2,511,224,510 |
| 4,977 | | 297,500,000 | 4,610,750,695 |
| 2,384 | | 11,111,111 | 295,508,271 |

Clearly, there is poor correlation between spore yield and either temperature or dosage. The spore yields, however, appeared unreasonably low, and it is believed that this is due to the fact that the determinations were made with dehydrated material and that aggregations of spores with the body tissues of the grubs were not completely broken up in the grinding process. Accordingly, in two subsequent tests, spore yields were determined from grubs killed but not dehydrated.

In one test, grubs were inoculated with one dosage and maintained at each of three temperatures: 66°, 76°, and 86° F. Forty grubs were so treated at each temperature, the inoculum being 521,000 spores per grub. The spore yields from the grubs which became diseased were as follows:

TABLE 12. SPORE YIELD RELATIVE TO REARING TEMPERATURES

| Temperature | Number[1] of spores per grub | Number[1] of spores per gram of body (live) weight |
|---|---|---|
| 66° | 814,814,814 | 4,166,429,938 |
| 76° | 1,691,176,470 | 8,542,310,430 |
| 86° | 1,407,692,307 | 7,349,102,440 |

[1] Based on average of five counts.

Here, again, there is no well-defined correlation between temperature and yield of spores. Not only was the yield at 66° much lower than at the other temperatures, but the incidence of disease among grubs held at this temperature was low, and the rate of development of the bacteria was greatly retarded. At the two higher temperatures, the yield was actually greater at 76° than at 86°, although, in view of the counting technique employed, the difference may not be actually significant, even though statistically so.

In the other test, grubs were inoculated at five dosage levels and maintained at one temperature (78° F.). Again, 40 grubs were used in each case, with the results as follows:

TABLE 13. SPORE YIELD RELATIVE TO INOCULUM

| Dose | Number[1] of spores per grub | Number[1] of spores per gram of body (live) weight |
|---|---|---|
| 31,081 | 1,422,413,793 | 7,189,166,570 |
| 15,541 | 1,304,687,500 | 7,289,571,460 |
| 7,318 | 1,319,444,444 | 7,505,135,090 |
| 3,855 | 1,556,250,000 | 8,216,390,010 |
| 2,079 | 1,220,703,125 | 6,057,078,020 |

[1] Based on average of four counts.

Again, there is no trend in spore yield, either on an individual or on a weight basis, which can be associated with the dosage of spores inoculated.

On the basis of these experiments, it may be concluded that, when grubs are incubated at temperatures which favor the development of the milky disease, the yield of spores of *B. popilliae* per diseased grub is independent of the actual temperature employed, the dosage of spores inoculated, or the body weight of the individual. The time of disease development is, of course, affected by the temperature of incubation and probably, to some extent by the dosage of spores inoculated.

## THE TRANSMISSION OF MILKY DISEASE

Although individual grubs may become diseased by ingesting spores placed in turf artificially, the successful use of the milky disease spore dust (or its equivalent) as a control measure depends upon the transmission of the disease from grub to grub and the consequent increase and spread of the bacteria from the original treated spot.

### Methods of Disease Transmission

The living diseased grub may serve as a source of infection for, as previously noted, it may be bitten by a healthy grub, the latter thereby swallowing a mouthful of blood containing the infective agent. Both vegetative and spore forms of the bacterium may be effectively transmitted in this way. Whether or not a dead grub containing *B. popilliae* in its vegetative form alone may serve to transmit the disease has not been tested. When grubs containing mature spores die, they soon disintegrate, thereby liberating the spores which become incorporated in the soil. The rate of decomposition might have a bearing on the rate of disease transmission and is undoubtedly affected by the moisture conditions and the micro-flora and -fauna of the soil.

### Relative Effectiveness of Different Spore Sources

The relative effectiveness of different spore sources in causing disease was tested in the following manner. As a control, 100 grubs were reared in 100 cubic inches of sterilized soil to which grass seed was added to provide food. In the test groups, 98 healthy grubs were placed in 100 cubic inches of similar soil, and the inoculum was added in the forms listed in Table 14. In all groups, the inoculum was placed in the same spots relative to the healthy grubs. The grubs at the start were placed uniformly over the area of 100 square inches (depth of soil being one inch), but their movements were not restricted by any barriers. The grubs were thus incubated for ten days at 78° F. They were then removed from the soil, but immediately replaced in the same soil with barriers limiting the individual grubs to a space of one cubic inch. This was done in order that any secondary infection, due to the transmission of the bacteria by grubs early acquiring the disease, would not confuse the results. One week later the incidence of disease among the living grubs was determined, as follows:

Table 14. Infection of Grubs from Different Spore Sources

| Case No. | Source of spores | Per cent of grubs infected |
|---|---|---|
| 1 | 2 third instar grubs, incubation phase of disease | 4 |
| 2 | 2 second instar grubs, disease complete | 14 |
| 3 | 2 third instar grubs, disease complete (inoculated two weeks prior to test) | 15 |
| 4 | 2 third instar grubs, disease complete (diseased for over one month) | 14 |
| 5 | 2 third instar grubs, disease complete, grubs killed with cyanide | 16 |
| 6 | Blood from 4 diseased grubs placed in two spots | 43 |
| 7 | 10 grams of soil in which 2 diseased grubs had disintegrated, placed in two spots | 53 |
| 8 | Control, no inoculum | 0 |

These data suggest that the inoculants may be grouped into three categories which cause a low, intermediate and high incidence of disease. As might be expected, grubs containing only vegetative rods (case 1) transmitted the smallest amount of disease. Intact grubs[1], whether alive or dead, containing mature spores (groups 2, 3, 4, 5), were responsible for an intermediate amount of disease, while the highest incidence of disease resulted from spores which were in direct contact with the soil (groups 6 and 7). Spore dust was not tested, as equivalent dosages would have required about 40 grams. This would not only have been too bulky but, as concentrated masses, would probably inhibit free movement of grubs through it and ingestion of the contained spores.

This experiment was repeated in essentially the same way, but fewer inoculants and fewer grubs were used in the same amount of soil. Only 63 healthy grubs were used with half the inoculum used in the previous test. Sixty-four grubs served for a control. The inoculants used and the resultant incidence of disease are indicated in Table 15.

Table 15. Infection of Grubs from Different Spore Sources

| Inoculum | Per cent of grubs infected |
|---|---|
| 1 grub, incubation phase of disease | 0 |
| 1 grub, completion phase of disease | 0 |
| 5 grams of soil in which 1 diseased grub had disintegrated | 35 |
| Control, no inoculum | 0 |

The results of this test give little information, but do serve to emphasize the greater effectiveness of spores in actual contact with the soil. The lower incidence of disease presumably reflects both the lower population density and the reduced inoculum.

[1] These grubs did not necessarily remain intact during the course of the experiment.

### Effects of Grub Population and Inoculum Potential on Disease Transmission

Communicable diseases spread most rapidly in a concentrated population of susceptible individuals, and it is to be expected that the milky disease will build up more rapidly the heavier the beetle grub population. Evidence for this can be seen in the following laboratory test, in which both the grub population and the inoculum potential were varied. Populations of 100, 50 and 25 grubs were reared in 100 cubic inches of soil (100 square inches, one inch deep) to which grass seed had been added to provide food. In each population group, one, four and 16 grubs, diseased in the completion stage, constituted the inoculum potential. After 20 days, the resulting incidence of disease was recorded as in Table 16.

Table 16. Effect of Grub Population and Inoculum Potential on Infection of Milky Disease

| Original population | | | Population after 20 days | | |
|---|---|---|---|---|---|
| No. healthy grubs | No. diseased grubs | Per cent diseased | No. healthy grubs | No. diseased grubs | Per cent diseased |
| 99 | 1 | 1 | 27 | 41 | 60 |
| 96 | 4 | 4 | 32 | 47 | 59 |
| 84 | 16 | 16 | 10 | 60 | 86 |
| 49 | 1 | 2 | 35 | 4 | 10 |
| 46 | 4 | 8 | 17 | 23 | 58 |
| 34 | 16 | 32 | 4 | 19 | 83 |
| 24 | 1 | 4 | 16 | 3 | 16 |
| 21 | 4 | 16 | 4 | 15 | 79 |
| 9 | 16 | 64 | 2 | 12 | 86 |

It should be noted that while the actual inoculum potential (one, four, 16 diseased grubs) was the same for each population level, the number of diseased grubs relative to the number of healthy grubs at the start of the experiment was far greater at the lower population levels. This, together with the fact that the number of individuals involved in the lower population levels is less, makes it difficult to evaluate the data on a statistical basis. Moreover, during a 20-day rearing period, there would be an opportunity for a secondary infection to be realized. That is, the first grubs to become infected could in turn serve to infect other, healthy, grubs. Nevertheless, the increased incidence of disease relative to the higher inoculum potential is clearly evident and, if the infection is considered in terms of the percentage of diseased grubs present (i. e., the increase in percentage diseased), the incidence of disease increases progressively with the heavier population. This experiment indicates, however, that a heavy population can compensate for a low inoculum potential and, conversely, a heavy inoculum potential can compensate for a low population in causing a resultant high incidence of milky disease.

Another laboratory test of the contagion factor was made similar to the one discussed above. In this case, the inoculum potential was kept uniform relative to the soil area, but the population of grubs was varied. In order that a more or less equal number of grubs would be tested at each population level, larger soil areas were used for the smaller population. One grub containing mature spores constituted the inoculum for each 100 square inches of soil, one inch deep. Healthy grubs at the rates of 80, 26, eight and three per 100 square inches were introduced and incubated for ten days at 78° F. The grubs were then removed and replaced in sterile soil, isolated from each other, and incubated for another ten days before the incidence of disease was determined. This method of handling prevented additional infections arising from grubs early acquiring the disease. The results obtained are indicated in Table 17.

Table 17. Effect of Grub Population on Rate of Infection

| Area of soil, 1 inch deep | Inoculum | | After incubation | | |
|---|---|---|---|---|---|
| | No. diseased grubs | Healthy grubs placed | Total grubs present | Grubs diseased | Per cent diseased |
| 100 sq. in. | 1 | 80 | 53 | 10 | 15.9 |
| 300 | 3 | 78 | 70 | 4 | 6.1 |
| 900 | 9 | 72 | 70 | 0 | 0.0 |
| 1,500 | 15 | 45 | 41 | 1 | 2.4 |
| 100 | 0 | 81 | 66 | 0 | 0.0 |

This only adds further weight to the conclusion that the milky disease is contagious in the sense that diseased grubs may promptly pass on the disease to healthy individuals, and that this occurs to a greater extent with an increasing density of population.

In the laboratory tests discussed above, the time during which infection could occur was limited in order that the incidence of disease would primarily measure the effect of more or less direct transmission of the disease from grub to grub. Further communicability of the disease results from the subsequent death of the diseased grubs and the consequent increase in the spore population in the soil. The rapidity with which this occurs is affected by the original rate of infection as determined by the population of grubs and the inoculum potential of the bacteria, the feeding intensity of the grubs, the mortality rate of the diseased grubs, and the rate of body disintegration following death.

### Infection Rate Among Grubs Incubated with an Increasing Inoculum Potential.

In a number of tests on third instar grubs, a trend of increased disease could not be defined because the original infection rate was so low that most of the grubs pupated before the disease became well es-

tablished. In one case, however, the disease did increase rapidly and its trend can be followed. Ninety-nine healthy, field-collected grubs were removed from cold storage where they had been kept, and placed with one diseased grub in 100 cubic inches of sterilized soil to which grass seed was added to provide food for the grubs. The grubs, incubated at 78° F., were examined at weekly intervals, each time being replaced in the same soil, to which water and grass seed were added as needed. The results of the observations are tabulated below:

TABLE 18. INCIDENCE OF DISEASE RELATIVE TO AN INCREASING INOCULUM POTENTIAL

| Time, weeks | Number of grubs present | Number of grubs diseased | Per cent of grubs present diseased |
|---|---|---|---|
| 0 | 100 | 1 | 1 |
| 1 | 96 | 1 | 1 |
| 2 | 87 | 7 | 8 |
| 3 | 81 | 10 | 12 |
| 4 | 68 | 23 | 34 |
| 5 | 51 | 30 | 59 |
| 6 | 28 | 19 | 68 |
| 7 | 16 | 10 | 63 |
| 8 | 8 | 4 | 50 |
| 9 | 4 | 2 | 50 |
| 10 | 2 | 0 | 0 |
| 11 | 1 | 0 | 0 |
| 12 | 0 | 0 | 0 |

Of this group, only one grub pupated (after six weeks) and emerged as an adult. All others became diseased or for some other reason died before completing their development. The total number of grubs diseased is not known, as any given count includes some grubs that had been diseased at the time of the previous examination. Although there is no doubt that a large proportion did acquire the disease, it can be seen that in spite of an increasing number of spores present in the soil, some few grubs failed to become infected. But even these died before pupating. It may be that their feeding intensity was too low to permit either their pupating or acquiring the disease. The upward trend in the incidence of disease, at least until it reached a maximum of 68 per cent, is of such a nature as to suggest a possible logarithmic increase in the number of spores available as inoculum. It should be noted that the per cent of grubs diseased, based on the number present at the time of observation, rises to a peak and then declines, in spite of a progressive increase in spores due to the disintegration of diseased grubs.

In this experiment, the increase in spore number was terminated by the decimation of the supply of susceptible grubs within the area observed. A second experiment served to indicate the trend where a lack of susceptible grubs was not a limiting factor. The experiment was conducted just like the previous one except that, at the time of each

weekly observation, healthy field-collected grubs which had been kept in cold storage were added to replace those which had succumbed the previous week. In other words, at the beginning of each week, 100 grubs were present in the 100 cubic inches of soil used in the test. After the sixth week no further additions were made, for by this time the accumulation of dead grubs and their decomposition products together with putrefactive bacteria created an unfavorable environment. The results are indicated in Table 19.

TABLE 19. INCIDENCE OF DISEASE RELATIVE TO AN INCREASING INOCULUM POTENTIAL

| Time, weeks | Number of grubs present | Number of grubs diseased | Per cent of grubs present diseased | Number of healthy grubs added |
|---|---|---|---|---|
| 0 | 100 | 1 | 1 | |
| 1 | 78 | 1 | 1 | 22 |
| 2 | 84 | 9 | 11 | 16 |
| 3 | 78 | 34 | 44 | 22 |
| 4 | 74 | 38 | 51 | 26 |
| 5 | 64 | 37 | 58 | 36 |
| 6 | 45 | 21 | 47 | 55 |
| 7 | 34 | 11 | 32 | |
| 8 | 11 | 5 | 45 | |
| 9 | 4 | 4 | 100 | |
| 10 | 2 | 2 | 100 | |
| 11 | 1 | 1 | 100 | |
| 12 | 0 | 0 | | |

This test demonstrates a trend observed in the preceding test, namely, that even though there is a progressive increase in the spore content of the soil, due to the contribution made by the death and disintegration of diseased grubs, there is not a corresponding increase in the incidence of disease as judged solely by the per cent of grubs diseased at the time of observation. Instead, the incidence of disease increases to a point and then declines and, in this instance, a second rise followed.

The rise and fall of the percentage of grubs diseased, observed in both of the above tests, is due to the slow action of the disease in causing death. At the beginning of the test, the rate of infection increases more rapidly than the death rate due to milky disease. There comes a point, however, where the death rate is more rapid than the rate at which the remaining healthy grubs become diseased and, as a consequence, the incidence of disease, as judged by the per cent of grubs infected, declines. In the observations recorded in Table 19, the increase in death rate is obvious from the number of grubs that had to be added each week to replace the dead ones. Not all of the deaths were due to milky disease but, as indicated above, some were due to the unfavorable environment created by the decomposition of so many grubs. Had this not been a factor, a continuation of the experiment would undoubtedly have demonstrated a periodic or, at least, a poly-

modal, curve with, presumably, a decreasing amplitude. The reasons for this are that, with an increasing death rate due to milky disease and the addition of healthy grubs, the percentage of diseased, living grubs would decline. This would reach a low point, and then the rate of infection among the added grubs would again exceed the death rate to initiate a new curve (unless other facts interfered with the infection). A stabilized condition could be reached only if the death rate equalled the rate of infection.

An attempt was made to evaluate the effects of inoculum potential and population density under field conditions. In a turf area where the grub population was estimated on the basis of ten square foot samples to be about 3.3 grubs per square foot, nine plots were laid out. Each plot contained 25 square feet and was bounded by strips of heavy tar roofing paper set vertically in the ground to prevent migration into and out of the plot. During the first week in May, 1944, grubs were added to six of the plots, leaving three with an undisturbed population. To three of the plots, 15 grubs per square foot were added and, to the other three plots, 45 grubs per square foot were added, introducing the grubs below the surface of the ground. This presumably provided three population levels of 3.3, 18.3, and 48.3 grubs per square foot. On May 8, all plots were treated with spore dust supplied by the Bureau of Entomology and Plant Quarantine. Three dosages were applied at each population level at the rates of 1,250,000,000, 2,500,000,000, and 5,000,000,000 spores per square foot. These applications were exceedingly heavy, the largest being equivalent to about 4,808 pounds of spore dust per acre. In treating the turf, the spore dust was applied to the surface as uniformly as possible by means of a flour sifter.

By the middle of June, the three plots containing the heaviest grub population showed severe feeding injury, indicating that even the excessive dosages of spores applied did not act quickly enough to reduce the numbers of grubs to a point where obvious feeding injury did not occur.

All plots were examined when the first adult beetles were observed in the general region in which the plots were located. On June 29, soil samples were taken from each plot by means of an instrument used for cutting cup plugs in golf greens. Samples were taken to a depth of four inches, keeping each inch level separate. Approximately 75 cubic inches at each level from each plot constituted a sample. The presence of any Japanese beetle grubs or pupae in the samples was noted, and the remaining soil in the plots was examined for all stages of the beetle. The turf was pulled up and thoroughly torn apart, the underlying three inches of soil were sifted, and the soil to a depth of two more inches was carefully scratched with a small hand cultivator to recover all possible individuals. These were taken to the laboratory and examined for disease.

It is possible that a few adult beetles escaped from the plots before examination was made, but it is believed that the number was negligible.

The number of beetles and their developmental stages found, and the incidence of disease observed are indicated in Table 20.

TABLE 20. SURVIVING POPULATION IN TREATED PLOTS AFTER ONE FEEDING SEASON

| Estimated original population per plot | Spore treatment per plot | | | | | |
|---|---|---|---|---|---|---|
| | 31,250,000,000 | | 62,500,000,000 | | 125,000,000,000 | |
| | Total | Diseased | Total | Diseased | Total | Diseased |
| | 1 adult | 0 | 8 adults | 0 | 1 adult | 0 |
| | 16 pupae | 0 | 21 pupae | 0 | 3 pupae | 0 |
| | 45 grubs | 0 | 41 grubs | 1 | 10 grubs | 0 |
| 82 | 62 | 0 | 70 | 1 (1.5%) | 14 | 0 |
| | 28 adults | 0 | 26 adults | 1 | 30 adults | 0 |
| | 36 pupae | 0 | 30 pupae | 0 | 29 pupae | 0 |
| | 34 grubs | 11 | 38 grubs | 5 | 34 grubs | 14 |
| 358 | 98 | 11 (11%) | 94[1] | 6 (6%) | 93 | 14 (15%) |
| | 16 adults | 0 | 7 adults | 0 | 4 adults | 0 |
| | 80 pupae | 1 | 75 pupae | 3 | 22 pupae | 0 |
| | 173 grubs | 75 | 139 grubs | 39 | 66 grubs | 34 |
| 1,208 | 269 | 76 (28%) | 221 | 42 (19%) | 92[1] | 34 (37%) |

[1] Sometime between June 19 and June 29, these two plots were visited by moles, which presumably accounted for some reduction in beetle population, particularly in the high dose-high population plot.

These data indicate the incidence of disease only at the time of observation, and do not take into account the individuals previously killed by the disease. On the other hand, there is no justification for attributing all the mortality to the disease. Nevertheless, several conclusions can reasonably be drawn from these data. It is obvious that within the limits of this experiment, the population has a greater bearing on the results than has the initial inoculum. Even at the excessive dosages of spores employed, the plots with few grubs showed little disease present. At the higher population levels, the incidence of disease does not show as orderly an increase with increased dosage as might be expected. It is presumed that greater differences in the original inoculum would have resulted in a more pronounced graded response.

The mortality can be seen to increase in the plots containing heavier populations and, in two plots (the low population-high dosage plot and the high population-high dosage plot), the mortality seems unusually high considering the trends indicated by the other data. Increased mortality with increased dosage is less obvious than with increased population, but it is indicated in the three plots containing

the heavy populations. Some estimate of the role of the disease in contributing to this mortality can be obtained by a bioassay of the soil in which the grubs fed.

It was mentioned above that soil samples were taken at the time the plots were examined. Six or more samples were taken at random from each plot. The soil of the samples at each level from each plot was pooled and thoroughly mixed. Grubs known to be free of disease were then introduced into the soil, to which grass seed was added to provide food for the grubs. After incubation for 17 days at 78° F., the grubs were examined for disease, the incidence being as indicated in Table 21.

Table 21. Assay of Soil from Test Plots

| Depth of soil | Original population per sq. ft. | Low inoculum | Medium inoculum | High inoculum |
|---|---|---|---|---|
| | | | per cent of grubs infected | |
| 1 inch | 3.3 | 32 | 15 | 13 |
| 2 | | 7 | 11 | 0 |
| 3 | | 0 | 0 | 11 |
| 4 | | 10 | 0 | 4 |
| 1 inch | 18.3 | 68 | 80 | 96 |
| 2 | | 7 | 77 | 37 |
| 3 | | 36 | 44 | 8 |
| 4 | | 57 | 15 | 17 |
| 1 inch | 48.3 | 77 | 88 | 77 |
| 2 | | 24 | 31 | 72 |
| 3 | | 3 | 60 | 66 |
| 4 | | 3 | 29 | 33 |

It should be noted that, where the grubs are numerous, the diseased individuals are responsible for carrying the disease down in the soil. With the light populations, there is a greater tendency for the spores to remain near the surface.

Because of the fact that the incidence of disease is a graded response to the concentration of spores present in the soil, the spore population may be estimated by comparison of the response of test grubs with the response of similar grubs reared in soil containing known numbers of spores. The major difficulty in such a method of bioassay is the choice of a "standard" with which the unknown can be compared for, if interest lies in the probable number, the spores in both should have the same potency. This, obviously, is difficult to assure. In the present case, the spores present in the test soil can be considered to be from two sources, (1) those originally applied in dust form and (2) those produced in the grubs themselves and liberated into the soil following the death of the hosts. An experiment already reported, however, indicated that the potency of fresh spores

might be six times as great as that of spores in dust form. Consequently, an overestimate of the spore content of the unknown soil would be made if soil containing spore dust was used as a standard, and an underestimate might be made if fresh spores were used as a standard. Under such circumstances, a curve based upon the combined data of several tests such as illustrated by Beard (2) probably serves as a better standard than any one single series, unless the potency of the spores in the test is known to be the same as that in the soil used as a standard. It goes without saying that the time factor should be the same in any assay. In tests reported here, 17 days was arbitrarily chosen since, at the temperature employed (78° F.), the disease requires about 12 days to complete its development after introduction of spores in the host. An additional five days was allowed for ingestion of spores. Obviously, all grubs do not ingest effective doses at the beginning of the exposure period, and all stages of the disease may be found in grubs. This necessitates blood examination of the test insects to determine all positive cases.

Using a combined curve derived from three tests, the spore content of the nine plots under consideration was estimated. In all plots, the spore content present in the top four inches of soil showed an increase over the amount placed as spore dust, indicating a contribution by diseased grubs. On the basis that each dead, diseased grub contributed two billion spores, the increase in spore content was sufficient to account for the observed mortality of grubs in the three plots with the intermediate population (18.3 grubs per square foot), but was inadequate to account for the heavier mortality in the three plots containing the population of greater density (48.3 per square foot). Also, in the plot containing the low population of grubs treated with the high dose of spores, the increase in spore content was insufficient to account for the reduction in number of grubs from 82 to 14. The mortality in the other two plots at this population level could be accounted for by milky disease.

This assay adds weight to the conclusion that the host population has a greater effect on the resultant incidence of disease than does the size of the inoculum. It further indicates that heavy populations may also be subject to other mortality factors which might be confused with the milky disease factor, if care is not taken to segregate the latter. This second conclusion is emphasized by the results of the following test.

On July 29, August 1 and August 3, 1944, 96 four-inch flower pots were each filled with one pint (by measure) of moist, sterilized soil. These were divided into two equal series. In one series, five, ten or 20 Japanese beetle eggs were placed in each pot and in the other, ten, 30 or 90 eggs were placed in each pot. In other words, each of 16 pots had five, 20, 30 or 90 eggs, while each of 32 pots had ten eggs. Equal amounts of grass seed were sown on the surface of the soil in each pot. At each egg population level, spore dust was applied in three dosages, of two grams, one gram, and 0.5 gram per pot. Un-

treated checks were included. Four replicates of each dosage at each population level were provided in both series. After the grass had sprouted, all pots were plunged in soil outdoors and covered with cheesecloth to provide partial shade. The pots were watered as needed. After 11 weeks, the soil in each pot was carefully sifted and the grubs counted and examined for disease. Of a total of 276 grubs recovered, only one was diseased, indicating that the disease did not "take". Since almost all of the grubs found were only in the second instar, it is believed that low feeding intensity was responsible for the absence of disease. But the survival of grubs relative to the original population of eggs and dosage of spores is of some interest. Pooling the number of survivors in the four replicates in each case, the data are tabulated in Table 22.

TABLE 22. SURVIVAL OF GRUBS REARED FROM DIFFERENT EGG POPULATIONS IN POTTED SOIL TREATED WITH SPORE DUST

| Number of eggs per pot | Total eggs | No. of grubs present after 11 weeks Grams of spore dust per pot | | | |
|---|---|---|---|---|---|
| | | 2 | 1 | 0.5 | 0 |
| 90 | 360 | 12 | 9 | 12 | 14 |
| 30 | 120 | 27 | 16 | 9 | 13 |
| 10 | 40 | 17 | 20 | 12 | 17 |
| 20 | 80 | 18 | 7 | 4 | 11 |
| 10 | 40 | 10 | 4 | 12 | 8 |
| .5 | 20 | 2 | 7 | 8 | 7 |

It is obvious that the mortality is greater in the higher populations, regardless of the disease. In fact, it almost appears that, in a given space, a limited number of grubs will develop, regardless of the original number of eggs present. It is well known that under crowded conditions the grubs nip each other, but the effect of this on mortality is not known. It is very probable in nature that excessive populations will not maintain themselves. If the milky disease does not reduce the number, some other factor or factors will.

## FATE OF SPORES IN THE SOIL

In many areas, the program of spore dust distribution has been somewhat on a geographical basis, in some cases being more or less independent of the Japanese beetle grub population. Where the population was very low, or even absent, the application of spore dust was believed to assure infection when the beetle did appear or increase in numbers. In Connecticut, at least, application of spores in advance of the insect has not proved effective in preventing beetle increase, nor even in checking serious increasing infestations. Such failure can easily be explained in terms of inoculum potential and density of the host population, but what happens to spores of the milky disease in soil not, or lightly, infested by beetle grubs has some bearing on the problem.

## Vertical Distribution in Soil of Spores Originally Applied to Surface

An effort was made to trace the vertical course of spores in four soils having different physical characteristics. Since natural soils were desired, the test sites were by necessity in different locations and, consequently, subject to some differences in weather. Each plot consisted of 25 square feet and was bounded by a strip of tar roofing paper placed vertically in the ground. The four soil types can best be described by the data in Table 23.

TABLE 23. PHYSICAL CHARACTERISTICS OF TEST SOILS[1]

| Plot no. | Location | pH | Moisture equivalent | Per cent sands | Per cent silt | Per cent total colloids | Per cent clay |
|---|---|---|---|---|---|---|---|
| 1 | Windsor | 6.00 | 7.03 | 79.8 | 13.8 | 6.8 | 11.46 |
| 2 | Mt. Carmel | 5.15 | 17.75 | 55.0 | 24.6 | 26.6 | 20.40 |
| 3 | Cheshire | 6.00 | 19.20 | 52.8 | 31.2 | 33.8 | 16.00 |
| 4 | Mt. Carmel | 5.80 | 18.30 | 46.6 | 32.4 | 26.8 | 21.00 |

[1] As determined by the Soils Department of this Station.

On April 18, 1944, each plot was seeded with a lawn mixture, containing some rapidly germinating grasses, at the rate of five pounds per thousand square feet. Due to dry weather conditions, poor stands of grass were obtained on all plots with the exception of plot 3, in Cheshire. On May 22, 1,250 grams of spore dust were scattered as uniformly as possible with a flour sifter on each of the four plots. This dosage is equivalent to five billion spores per square foot of surface.

In plots 1 (Windsor) and 3 (Cheshire), no beetle grubs were present as judged by test diggings in surrounding areas. In plot 2 (Mt. Carmel), grubs were present to the extent of about one per square foot, whereas about three per square foot were present in plot 4 (Mt. Carmel).

At intervals of two weeks, six weeks and 18 weeks after the spores were applied to the surface of the soil, samples were taken to determine the disease-producing capacity of the soil. At each time, five or more samples were taken from each plot by means of an instrument used in cutting cup plugs in golf greens. A cylindrical core of soil was taken of the top four inches and divided into one-inch sections. The samples from each inch level from each plot were pooled, sifted through an eight-mesh screen, and thoroughly mixed in a small barrel-type mixer. Moisture and grass seed were added, and grubs were introduced. Forty isolated grubs were used in each sample. Since the assays were made on three different occasions, grubs were reared in soils containing known numbers of spores in serial dosages to provide standards for comparison. By using the three standard dosage-response curves, the probable dosage of spores in each unknown sample

could be inferred and, by reference to a common curve based on the combined data of the three standards, the disease-producing capacity of the soil could be corrected in terms of the different susceptibilities among the three lots of grubs used. The results obtained are indicated in Table 24.

TABLE 24. DISEASE-PRODUCING CAPACITY OF FOUR SOIL TYPES AT INTERVALS AFTER APPLICATION OF SPORES

| Plot | Depth of soil | Disease-producing capacity as found (Per cent of grubs diseased) | | | Disease-producing capacity corrected for differences in grub susceptibility (Per cent of grubs diseased) | | |
|---|---|---|---|---|---|---|---|
| | | 2 weeks | 6 weeks | 18 weeks | 2 weeks | 6 weeks | 18 weeks |
| 1 | 1″ | 44 | 29 | 18 | 54 | 69 | 16 |
| | 2 | 0 | 3 | 0 | 0 | 2 | 0 |
| | 3 | 6 | 0 | 0 | 7 | 0 | 0 |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 1″ | 19 | 33 | 5 | 23 | 76 | 9 |
| | 2 | 6 | 4 | 0 | 7 | 3 | 0 |
| | 3 | 0 | 13 | 0 | 0 | 26 | 9 |
| | 4 | 0 | 17 | 5 | 0 | 38 | 9 |
| 3 | 1″ | 24 | 38 | 23 | 29 | 84 | 18 |
| | 2 | 22 | 7 | 0 | 26 | 52 | 0 |
| | 3 | 0 | 3 | 3 | 0 | 2 | 7 |
| | 4 | 6 | 4 | 2 | 6 | 3 | 7 |
| 4 | 1″ | 16 | 29 | 23 | 19 | 70 | 19 |
| | 2 | 3 | 5 | 18 | 3 | 5 | 16 |
| | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 3 | 0 | 51? | 3 | 0 | 30? |

A peculiar aspect of these data is the increase in the disease-producing capacity of the soil tested six weeks after the application of spores. This might be accounted for in plots 2 and 4, if it were assumed that an occasional grub became diseased to contribute additional inoculum. This is unlikely, since plots 1 and 3 presumably harbored no grubs, and yet they, too, showed an increase—at least if the corrected data are considered. One explanation might be that, at the time of the first assay, a large part of the spores were adherent to the grass, which was removed when the samples were sifted. By the time of the second assay, the spores could have been washed into and become a part of the soil. Another possibility is that the assay itself overestimated the disease-producing capacity of the soil. This could happen if the grubs used in the unknown soils were more susceptible than the grubs reared in the soils containing known numbers of spores. In view of the techniques employed, it is doubtful if such systematic differences could be explained in this way. Still another possibility is that the spores are actually favored by contact with the soil. In view of the subsequent decline, this seems improbable, although a suggestion of the same effect can be seen in a test earlier

described in which spores exposed to soil during a summer period caused somewhat more disease than similar spores not thus exposed.

In general, there seems to be a tendency for the spores to remain in the upper layers of the soil. It is possible that the sampling technique used, by allowing a small amount of mixing of the different layers, accounted for a little of the apparent vertical distribution of spores. But it is evident that some vertical spread does occur, and strangely enough this seems more pronounced in the heavier soils of plots 2, 3 and 4 than in sandy plot 1. The unusually high disease-producing capacity of the fourth inch sample of plot 4 in the 18-week assay is undoubtedly abnormal. In this case, it is very likely that one or more diseased grubs contributed additional inoculum to this soil.

After 18 weeks, there appears some loss in the capacity of the soil to produce disease. This seems to be more true of the lighter soils of plots 1 and 2 than of the heavier soils of plots 3 and 4. It may be that the soil colloids are an aid in fixing the bacteria to the soil and that the loss of effective spores is due more to a diluting or leaching factor than to an actual mortality of bacteria.

Except for the small differences noted, the soil types here observed had limited differential effects upon the fate of spores applied to the surface.

### Vertical Distribution in Soil of Spores Naturally Released

Some idea of the distribution of spores in soil in which the milky disease organism has increased naturally can be obtained from an assay of such soil. In a turf area of a golf course where the incidence of disease was known to have been high the previous year, soil samples were taken of the top three inches in April, 1944. Grubs reared for 17 days at 78° F. in these samples became diseased to the extent of 90 per cent in the top inch, 54 per cent in the second inch, and 46 per cent in the third inch. This confirms the conclusion that the spores tend to be more abundant in the surface layers of the soil.

## THE EFFECT OF MILKY DISEASE ON DEVELOPING POPULATIONS OF JAPANESE BEETLE LARVAE

The experiments discussed above were directed largely towards an understanding of the individual factors that affect the infection of Japanese beetle grubs by *Bacillus popilliae* and transmission of the organism from host to host. An integration of these factors may be observed to some extent when developing populations in the presence of the milky disease organism are closely observed.

### Developing Grub Population in the Presence of a Constant Inoculum

Although a natural grub population developing in infected soil increases the inoculum through the agencies of death and disintegra-

tion of diseased grubs, it is desirable to know the effects of the disease when the spore content of the soil is not increased. Such effects were observed in the following manner.

Japanese beetle eggs were obtained by enclosing adult beetles in quart jars containing a small quantity of moist soil. Food in the form of smartweed shoots or soybean leaves was supplied to the beetles daily. Eggs are readily deposited under such conditions and may be isolated by sifting the soil. The eggs were placed on moist filter paper in Syracuse watch glasses and incubated at a temperature of 86° F. Promptly upon emerging from the eggs, the larvae were placed in soil. Four soil preparations were used—sterilized soil with no addition of spores to serve as a control, and three lots of soil containing milky disease spore dosages of 446,000, 893,000 and 1,786,000 spores per gram, dry weight, of soil. Spore dust, supplied by the Bureau of Entomology and Plant Quarantine, served as the source of spores. The newly-emerged first instar grubs were placed in Petri dishes containing the prepared soil. At the beginning, 25 grubs were reared in one dish but, as grubs died, the amount of soil available per grub increased accordingly. At the time of the second ecdysis, the grubs were individually placed in Syracuse watch glasses for further rearing. Also, any grub becoming diseased, regardless of its instar, was isolated for observation. When it died, it was discarded so that the bacteria contained in its body would not be added to the inoculum as prepared. At all times, an abundance of food was provided and moisture conditions were maintained. For a period of about two and a half months, the grubs were reared at room temperature which ranged from a maximum of 92° to a minimum of 64°. During the latter part of the period, when lower temperatures pertained, the development of the grubs was so slow that all grubs were placed in a constant temperature room maintained at 78° for the remainder of the experiment.

The grubs were examined at five-day intervals, and note was made of the number of individuals, stage of growth, and presence or absence of disease, basing the latter upon macroscopic appearance and not upon microscopic examination of the blood. Individual records were kept for the grubs becoming diseased.

When the data were tabulated, it was found that the usual graded response to increased dosages was not present, but that in all three treated soils, the infection rate was not significantly different and was reasonably low. It is presumed that, because of low potency of the spores used, the dosages were inadequate to produce the graded response. As a consequence, the data for the three groups of grubs reared in soil containing spores of *Bacillus popilliae* are combined. In the control group, although the sterilized soil was replaced five times during the experiment as a precaution against contamination, some noticeable contamination did occur approximately 115 days after the beginning of the observations. After this time, a number of grubs within small groups became diseased, suggesting a sudden introduc-

tion of spores in concentrated form, such as might result from the addition of a diseased grub in disintegrated form. It is not known how this contamination was made. The amount of disease resulting before the contamination was eliminated accounted for 4 per cent of the mortality in the check group, but this came late enough in the experiment so that the major trends were unaffected. The only serious consequence is that the amount of pupation in the control group is probably somewhat less than it would have been otherwise. When a grub pupated, it was considered no longer subject to disease, although this is not strictly true, and was no longer kept under observation. Moreover, pupal mortality was not observed, so that the mortality noted in the summary below is limited to larval mortality. The experiment was terminated when all grubs had either died or pupated.

Although observations were made at five-day intervals, the data summary (Table 25) gives information on ten-day intervals, as such grouping renders some trends more obvious without loss of information. The experiment began with 425 control grubs and 1,710 grubs in the spore-containing soil. Molting trends and mortality trends of diseased grubs in the three instars as determined from data in this experiment have been discussed in a previous section.

It is apparent from the table that, under these conditions, the disease was by no means catastrophic, but it exercised a more or less constant depressing action on the population, becoming evident in the *gradual* increase in the cumulative mortality due to disease. The greatest mortality occurred in the first ten days of larval life, before the disease had a chance to be acquired. This is evident from the high initial mortality in the control grubs as well as the grubs reared in infected soil.[1] The observation that there is no particular time during the grub's existence when it is more susceptible than at other times confirms the test discussed in a previous section in which the three larval instars were found to have similar susceptibilities. From the percentage of grubs becoming diseased during each ten-day period, it may be inferred that, during periods of reduced feeding activity, such as were observed during molting and towards the end of the experiment, the infection rate was somewhat less than usual.

The lack of agreement between the effects of the disease as judged by the incidence of disease at the time of observation and the actual mortality attributable to the disease should be noted. Although at one time (100 days after hatching), 22.5 per cent of the existing grubs were diseased, 8.2 per cent of the original population had succumbed from the disease. Fifty days later, when 11.3 per cent of the original population had died following infection with milky disease, 7.9 per cent of the existing grubs were seen to be infected. More will be said in regard to the use of percentages in this way in the discussion below.

[1] Undoubtedly, much of this mortality was due to the method of handling, in allowing the grubs to hatch before placing in soil. In the experiment next to be discussed, eggs were placed in the soil and allowed to hatch without handling. The initial mortality under these conditions was much less.

TABLE 25. EFFECT OF MILKY DISEASE ON A GRUB POPULATION DEVELOPING IN THE PRESENCE OF A CONSTANT INOCULUM

| Days after hatching | Control grubs | Grubs reared in infectious soil | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cumulative mortality Per cent | Cumulative mortality Per cent | Cumulative mortality due to disease Per cent | Per cent disease at time of observation | Per cent of grubs becoming diseased (10-day period) | Per cent grubs in first instar | Per cent grubs in second instar | Per cent grubs in third instar |
| 10 | 68.2 | 64.7 | 0.0 | 0.2 | 0.2 | 99.8 | 0.2 | |
| 20 | 74.8 | 73.4 | 0.3 | 8.6 | 7.3 | 34.1 | 65.9 | |
| 30 | 78.8 | 78.6 | 2.2 | 6.6 | 4.7 | 4.7 | 95.3 | |
| 40 | 80.0 | 80.4 | 3.0 | 12.5 | 9.5 | | 80.4 | 19.6 |
| 50 | 81.9 | 82.9 | 4.0 | 14.7 | 6.2 | | 28.1 | 71.9 |
| 60 | 82.8 | 84.5 | 5.0 | 14.0 | 4.2 | | 15.5 | 84.5 |
| 70 | 83.8 | 86.4 | 5.9 | 14.7 | 5.6 | | 11.2 | 88.8 |
| 80 | 83.8 | 87.8 | 7.0 | 18.8 | 11.1 | | 9.6 | 90.4 |
| 90 | 84.2 | 88.8 | 7.6 | 20.4 | 5.8 | | 8.4 | 91.6 |
| 100 | 84.9 | 90.1 | 8.2 | 22.5 | 5.9 | | 7.7 | 92.3 |
| 110 | 86.6 | 91.3 | 9.1 | 21.3 | 6.0 | | 4.7 | 95.3 |
| 120 | 87.8 | 92.9 | 9.9 | 18.0 | 3.3 | | 3.3 | 96.7 |
| 130 | 89.9 | 94.8 | 10.8 | 12.4 | 3.4 | | 2.2 | 97.8 |
| 140 | 91.5 | 95.8 | 11.0 | 10.3 | 0.0 | | 1.5 | 98.5 |
| 150 | 94.1 | 97.1 | 11.3 | 7.9 | 2.6 | | | 100.0 |
| 160 | 94.6 | 97.8 | 11.5 | | | | | |
| 170 | | 98.1 | 11.5 | | | | | |
| 180 | | 98.7 | 11.5 | | | | | |
| 190 | | 98.9 | 11.5 | | | | | |
| | 5.4% pupated | 1.1% pupated | | | | | | |

**Developing Grub Population in the Presence of an Increasing Inoculum**

The above experiment was repeated in essentially the same way except that the dead grubs were not removed from the soil. This allowed the bacterial contents of the diseased grubs to be added to the original inoculum. Also, the eggs, instead of the newly-emerged larvae, were placed in the soil directly. This resulted in greater survival at the start of the experiment. No more than 25 eggs were placed in a Petri dish containing the desired soil. When placed, the eggs were no more than two days old. The first observation was made after two weeks, and examinations were made thereafter at weekly intervals. As before, as grubs died, more soil per grub became available, but no larger containers than Petri dishes were used, and the grubs were not isolated when they reached the third instar, as was done in the previous test.

Throughout the experiment, the grubs were reared in a constant temperature oven maintained at 86° F.

Two series of grubs were reared in two lots of soil containing spores of *B. popilliae*. A third group was reared in sterilized soil as a control. At the time of each observation, the grubs in the last group were not replaced in the same soil, but were placed in fresh sterilized soil. This was done to prevent contamination and the infection of the check grubs. Thus, not only were the check grubs reared in soil free from milky disease spores but, also, the dead grubs resulting from other mortality factors were not allowed to accumulate. For this reason, there is probably a greater difference in the mortality between the control grubs and those subjected to milky disease than if the presence of milky disease spores had been the only difference in the soil used for rearing.

In one group of grubs reared in infectious soil, the disease was initiated more promptly than in the other group. It would be expected that the disease would spread more rapidly and have a greater total effect in this group. Such was the case, although in both series no grubs survived to pupate, and the time required for complete mortality was about the same in both cases (Figure 26). As compared with the control series, little difference in mortality was apparent among the three groups until the seventh week after the eggs were placed. From then on, the effect of the disease on mortality became noticeable.

In the two groups of grubs reared in infectious soil, the rates of infection followed similar trends, but were of different magnitude (Figure 27). In both cases, the rate did not increase progressively but, as has been observed in other experiments, declined after a maximum had been reached. In the group showing a delayed effect of the disease, a final spurt from 0 to 100 per cent infection appeared. This is because the sole survivor became infected at that time. When the fate of the original population and the size of the existing population

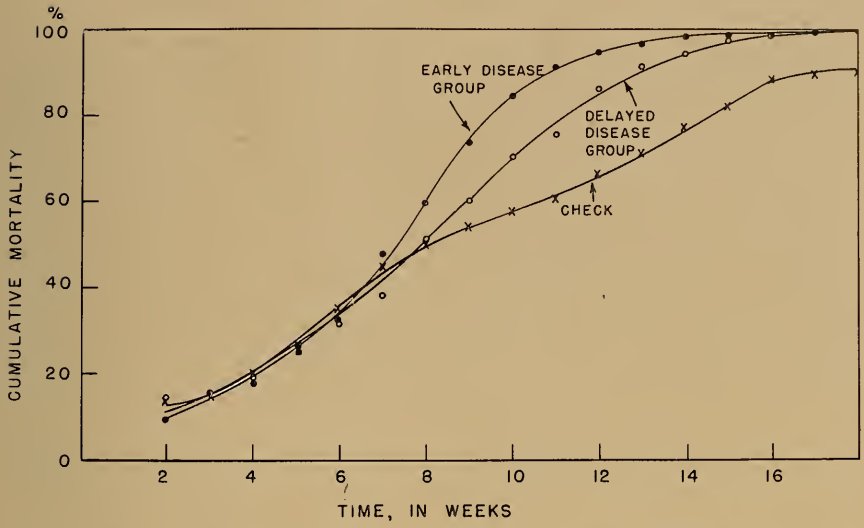


FIGURE 26. Cumulative mortality trends of two groups of grubs reared in infectious soil and one group reared in sterilized soil.

are known, as in these observations, little significance is attached to such a dramatic increase in the rate of infection. In the field, however, where data are often taken in terms of percentage infection without regard to the original population, the existing population, and the mortality factors acting upon them, misleading interpretations can be drawn from data not too dissimilar from these.

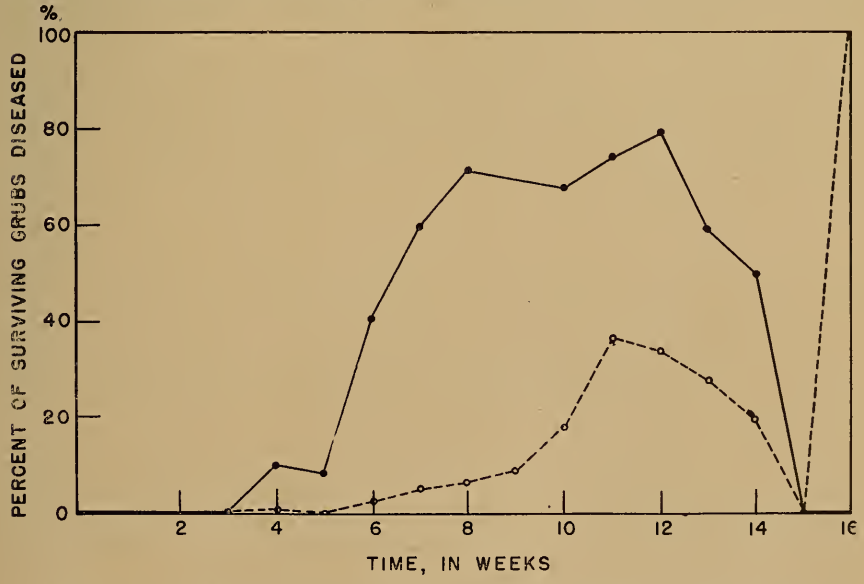


FIGURE 27. Infection rates of two groups of grubs reared in infectious soil.

It should be pointed out that the populations used in this experiment, in terms of grubs per unit of soil, were very heavy. If it is assumed that in the field an actively feeding grub population is confined to the upper two inches of soil, the populations in these tests were equivalent to approximately 1,200 eggs per square foot at the beginning of the experiment and, by the time mortality was complete in the two groups reared in infectious soil, the population in the check was still equivalent to over 100 grubs per square foot. Such crowded conditions are ideal for the rapid spread of the disease, particularly when favored by the high temperatures employed. In spite of such optimum conditions, however, a period of about four months was required to decimate completely the grubs in the infectious soil, and by no means all of the mortality was due to the milky disease alone.

## GENERAL CONSIDERATIONS OF THE RELATIONSHIPS BETWEEN *Bacillus popilliae* AND *Popillia japonica*

### *Bacillus popilliae* as a Parasite

In name and in use, the relationship between *Bacillus popilliae* and *Popillia japonica* has been emphasized as a disease. This tends to focus thought on the pathological effects of the invading organism. It has been shown, however, that, in the milky disease, these pathological effects are at present somewhat obscure, although they are manifest in the inhibition of molting and metamorphosis in the host and in the causing of premature death. It seems desirable, then, to shift the emphasis from the *effect* to the *cause*, and to give greater consideration to *Bacillus popilliae* as a bacterial parasite.

The origin of *B. popilliae* as a parasite of Japanese beetle larvae in the United States is of more than passing interest, for if this were known, some of the peculiarities of the organism could undoubtedly be explained and a more reliable concept of its potentialities as a control measure could be realized. Three possible origins of the bacterium can be postulated.

1. The parasitic bacteria could have been introduced from the Orient along with the host itself, which reached this country sometime prior to its discovery here in 1916.

2. The bacterium may have been parasitic upon other insects such as native scarabaeid larvae and have found the Japanese beetle larva a more favorable host, particularly when it increased in remarkable numbers.

3. The bacterium may not have been entomogenous originally, but had an independent existence, accidentally becoming parasitic in a facultative sort of way when the beetle grubs became very abundant.

Proof of any one of these may not be obtained, but certain considerations are worthy of attention.

Dutky (11) implied a belief that the bacterium was an introduced parasite, while Fox (18), without mentioning the milky diseases specifically, concluded that bacterial parasites of native scarabaeid larvae became parasitic in the Japanese beetle. Wheeler and Adams (43) stated that there was no evidence that the parasite was imported, but was presumed to be "derived from a type of bacterium harbored originally from some grub native in New Jersey".

From a practical control point of view, there is little fundamental difference whether the bacterium was originally a parasite in Japanese beetle grubs in Japan or in a similar, closely related native grub. On the other hand, if the bacterium was originally not entomogenous at all, but took up a parasitic existence, possibly by accident when the beetle grubs became numerous, a knowledge of such evolution would be of real practical value. If *B. popilliae* is an entomogenous parasite of long standing, it should have the characteristics of a parasite well adapted to its host. On the other hand, if its evolution to a parasitic form is very recent, it should manifest some characteristics peculiar to a free-living organism.

In many ways, *B. popilliae* shows evidence of being a parasite well adapted to its host. In the first place, it has a restricted portal of effective entrance, which in itself is an indication of a well adapted parasite. Moreover, its locus of infection is limited to a single tissue, the blood, without the formation of obvious lesions to tissues involved in the invasion process. Once the disease becomes established in the host, it almost assumes a chronic form since the host is not immediately incapacitated, but continues to feed for a more or less prolonged period. To be sure, development of the host is inhibited, and death comes somewhat prematurely, but the disease is not immediately lethal. Instead, the fatal effects are usually delayed until the parasite has completed its development. This low pathogenicity is perhaps the most cogent evidence that *B. popilliae* is a highly evolved parasite. Biologically, a true parasite lives at the expense of its host without causing lethal effects. It is to the parasite's advantage to avoid killing the host before its own life cycle is completed.

The postulate that the milky disease organism might be a free-living bacterium that accidentally invades Japanese beetle larvae when the latter become very numerous would not be unique. Babers (1), for example, reported that a common soil bacterium, *Bacillus cereus*, caused a septicemia in the southern armyworm (*Prodenia eridania* Cram.), being highly infectious and very pathogenic.

One peculiarity of the milky disease organism is its ability to sporulate before any lethal effects are caused in the host. Whether this is because the parasite is exceptionally well adapted to the host or whether this is characteristic of a free-living organism is difficult to interpret. Too few entomogenous diseases have been studied sufficiently from a pathological standpoint to draw comparisons, and it is probably dangerous to draw analogies from bacterial parasites of ver-

tebrates. In regard to the latter, Smith (38) has made the generalization that endogenous spore formation is frequent among free-living bacteria but, representing a definite cycle, is not known among parasitic forms. This conclusion is qualified somewhat by pointing out that the anthrax bacillus sporulates, but not in the living or dead tissues of the body. It is only after discharge to a suitable environment that spores form. Furthermore, other disease-producing bacteria which sporulate, such as the tetanus bacillus, and the bacilli causing gas gangrene (*B. welchii*), malignant edema (*B. edematis maligni*), and black leg in cattle (*Clostridium chauvei*) are not essentially parasites, but depend upon open wounds or other organisms for a stimulus to grow, and even these bacilli do not sporulate in the living host, but require anaerobic conditions produced after death. Such generalities should not be too strictly applied, since the line of demarcation between a parasitic form and a non-parasitic form is not well-defined.

Among entomogenous fungi, it has been implied by Fawcett (17) that obligate parasites sporulate after the death of the host.

From the information available at the present time, it would seem that the sporulation of the milky disease bacteria in the living host and the absence of any obvious effects of the disease until this occurs, is an unusual, if not unique, case.

Strict parasites have a tendency to infect hosts of a definite age. This may be due to different reasons in different forms and, consequently, may not be a good criterion for a parasite. It has been shown that all grub stages of the Japanese beetle are equally susceptible and, by injection, the disease can be induced even in the adult beetle (Dutky, 11, Langford et al., 26). In contrast, the larva of the honeybee is most susceptible to spores of American foul brood (*Bacillus larvae*) during the first day of larval life, and is probably not susceptible after two days (Woodrow, 51). Since this is associated with the period of mass feeding (Woodrow, 50), these differences in susceptibility may be due to the feeding habits of the two insects rather than the relative degree of adaptation on the part of the two bacterial parasites.

The infectivity of a well adapted parasite tends to be less than an accidental one, at least among vertebrate pathogens, largely because the customary host has developed resistance of some kind. This, of course, may not be true of insect diseases, and examples are scarce from which analogies may be drawn. Certainly, the infection of the southern armyworm by *Bacillus cereus*, an "accidental" parasite, reached epizootic proportions in the laboratory (Babers, 1). On this basis, the low rate of infection among Japanese beetle grubs exposed to spores of *Bacillus popilliae* would point to a well-established host-parasite relationship. In this case, low infectivity is doubtless associated with another factor already mentioned, namely, a restricted locus of effective entrance. Experimentally, however, this is not the limiting factor, for if the bacterial spores are injected directly into the body cavity where, presumably, the spores may germinate, a relatively

large number is required to induce infection. In this case, the probability of viable spores failing to reach an environment favorable to growth is reduced to a minimum, unless the spores do not germinate in the blood, but must be transported to some definite locus for germination, to return to the blood in the vegetative form. If the spores do actually germinate in the blood when placed there, some sort of resistance or immunilogical reaction must be present to account for the low infectivity of the organism and the dosage-response.

The fact that the milky disease has been observed in areas where no spores have been artificially introduced and at distances from treated areas precluding the usual natural agencies of dissemination can be explained either on the basis that the organism is an accidental parasite or that it is an established parasite of some native insect or insects.

Although nothing definite is proved, the above considerations point to the probability that *B. popilliae* is an obligate parasite of scarabaeid larvae, the grubs of the Japanese beetle being at present its most common host.

## Epizootiology of the Milky Disease

Disease epidemics frequently follow a pattern such that the morbidity of the disease, plotted against time, corresponds to the normal curve of error. The ascending portion of the curve is due to the contagion factor among a susceptible population, with a probable increase in virulence of the causal organism. The descending portion of the curve may be due to several factors, the most important being a decreasing number of susceptible persons because of natural or acquired immunity. Moreover, some epidemics exhibit a periodicity in that the epidemic curve is repeated from time to time. The factors iniating a new epidemic may vary with different diseases, but the epidemic frequently follows an accumulation of susceptible individuals. At any time, the host population may be said to be made up of infected individuals, individuals who are immune, and susceptible individuals. With some diseases, the immune group represents those who have recovered from an attack of the disease.

If the milky disease of Japanese beetle larvae caused epizootics comparable in form with epidemics which can be represented by periodic normal curves, its usefulness would be limited. We could then expect no permanent protection against the beetle grub in a treated turf area and, at best, could hope for a partial, temporary and localized reduction of the grub population.

Fortunately, one factor stands in the way of such a situation, and that is the inability of an infected grub to recover from the disease and thus establish an immunity. It is possible that the resistance that has been noted may have selective value in developing a race of Japanese beetles, the larvae of which are resistant to the disease in a way

comparable to the races of codling moth resistant to the action of lead arsenate. At present, it appears that most of the disease resistance, whatever its nature, can be overcome by an increase in the inoculum. However, the accumulation of the more resistant individuals is, undoubtedly, at least partially responsible for the decline in the incidence of disease noted in several experiments discussed in the previous section. In actual numbers, these "resistant" individuals may be very few but, when the incidence of disease is expressed on a percentage basis, their importance may be exaggerated.

The morbidity curve of milky disease, here referred to and noted in several tests of different natures, superficially resembles the normal epidemic curve in that the disease incidence rises to a peak and then falls. The drop in incidence, however, instead of being due to the lack of susceptible individuals, is a phenomenon associated principally with the relative rates of infection and mortality. Undoubtedly, the accumulation of the more resistant grubs affects the trend, but this is not the factor most responsible for the observed tendency. It has already been suggested that a periodic, or at least polymodal, curve of morbidity could possibly be demonstrated under experimental conditions. Certainly, in nature, a periodicity occurs, for each year a new generation of susceptible individuals is introduced and, in this way, the disease is comparable with some epidemics. The rise and fall of the morbidity rate is not necessarily characteristic of the infection during each feeding season, but it has been observed in the field.

The important difference between the milky disease epizootic and some of the common epidemics is that the hosts classified as diseased are removed from this category by death in the former, and by recovery (usually) in the latter. For this reason, even if the trends appear similar, the results may be quite different.

Most field data concerning the milky disease are recorded in terms of the percentage of grubs diseased at the time of observation, correlated, where possible, with the reduction of population as judged by comparisons with check areas. This is necessitated by the fact that, at any observation, it is impossible to know how many grubs have already succumbed to the disease, died from other causes, or, at certain times, how many have escaped infection by pupation and emergence. It has been seen, however, that a high incidence of disease may be evident when the fatal effects are low. At other times, the percentage of infected individuals may be low when the disease has wiped out a large proportion of the original population. In other words, the incidence of disease at any given time may bear very little relation to the actual contribution of the disease in causing mortality. It is also possible to observe an apparent, but misleading, high rate of infection late in the spring when the insects are pupating and emerging as adults. Since the diseased grubs are unable to pupate, they may seem to predominate due to the fact that the healthy grubs have completed their development and escaped. In the same way, a misleading conclusion could be drawn at the time the bulk of the young grubs are

molting to a later larval instar. Here again, the younger instar might be represented chiefly by diseased individuals, and the conclusion drawn that the younger grubs were more susceptible to the disease when, as a matter of fact, the healthy grubs molted, leaving the residue of diseased grubs in the earlier instar.

It is quite possible that, under certain conditions where the population is very heavy, secondary effects of the disease might be responsible for increased mortality that could be attributed erroneously to the direct effect of the disease. That is, in heavy populations, the disease could kill effectively a sufficient number of grubs so that the accumulated decomposition products and putrefactive bacteria themselves contributed to a decline of the grub population. Although these factors have not been studied objectively, there is evidence to indicate that an accumulation of dead grubs in the soil, regardless of the cause of death, creates an environment unfavorable, not only for the development of the living grubs contained there, but also for the development and spread of the disease. Although such a condition may not be encountered frequently, it merits further study and experimentation.

In view of the above considerations, it might be possible to evaluate more satisfactorily the effects of milky disease in the field by means of a bioassay of the soil. The effectiveness of such soil in causing infection among healthy grubs is a measure of the number of viable spores present and reflects the cumulative mortality due to the milky disease. Certain aspects of assay methods have already been discussed, but the usefulness of such a procedure for the evaluation of field plots would depend upon suitable sampling methods for the soil as well as the technique employed in the assay.

## SUMMARY

The milky disease of the Japanese beetle, caused by a bacterium, has become widely known in the field of biological control of insects. The discovery of the disease and the development of methods for utilizing it in the field against the larvae of the Japanese beetle have been due to the work of the Bureau of Entomology and Plant Quarantine of the United States Department of Agriculture. Extensions of this work have been made by cooperating agencies in various states in the regions infested by the Japanese beetle. The available published material is reviewed as a background for the present studies.

The causal organism of milky disease (Type A), *Bacillus popilliae* Dutky, is a slender, non-motile rod in its vegetative form, and forms spores of characteristic shape.

The normal invasion route of the bacterium is via the alimentary tract of the host. Both the vegetative and spore forms of the bacterium seem to be infective, but the spores apparently germinate before reaching the locus of infection, the blood (haemolymph). Al-

though not conclusively proved, evidence indicates that the Malpighian tubules are the most probable site of bacterial penetration from the gut to the blood.

For purposes of discussion, the course of the disease in the host is divided into four developmental phases: invasion, incubation, sporulation and completion. The time required for the disease to develop is largely a function of the temperature.

Infected grubs do not necessarily die when the diseased condition is complete, but may live—and feed—for a longer or shorter time, presumably depending on the vigor of the individual grub. Diseased young grubs die sooner than diseased older grubs. Although the disease does not cause prompt death, it does inhibit molting and metamorphosis of the host.

The bacteria develop solely in the blood of the grub, and there is no apparent disturbance in the other body tissues or organs. Changes in the inorganic chemical constitutents of the blood, the number of blood cells, the osmotic pressure of the blood, blood pH, or manner and time of blood coagulation are too slight to account for the over-all effect of the disease. It has been found, however, that the disease does commonly disturb at least one oxidizing enzyme system and, since it is probable that oxidizing enzymes are necessary for molting, metamorphosis and full realization of life expectancy, it is believed that the effects of the disease may be caused by the destruction of one or more enzyme systems.

The probability of a grub becoming infected increases with the spore dose, whether received by injection into the body cavity or by ingestion into the gut with food.

The three larval instars appear equally susceptible.

As tested, grubs fed prior to inoculation showed susceptibilities not differing significantly from grubs removed from cold storage at time of inoculation.

Grubs incubated at temperatures of 75° and 85° F. appear equally susceptible to milky disease induced by inoculation.

Spores kept as dried blood films show no loss of potency with time.

As here tested, exposure of spores in soil to weather caused no loss in potency.

A single test indicated that fresh spores were six times as potent as spores in dust form.

Exposure of spores to ultraviolet light (from sun lamp) caused very marked reduction of potency.

Although low pH appears to affect spores of *B. popilliae* adversely, the pH of soils usually encountered in Connecticut does not affect significantly the potency of spores.

Spores show marked loss of potency when heated at temperatures above 194° F.

The effect on potency of successive passages of the bacteria through a series of hosts is not well defined. Increased potency has been observed, but this has not been maintained consistently.

Some loss of potency has been observed when spores were kept refrigerated in a water suspension.

The average number of spores produced per grub approximates two billion. The number of spores produced is not correlated with the body weight of the host, the temperature of incubation (provided it is favorable), nor with the size of inoculum.

Healthy grubs may acquire the disease by biting diseased individuals. More commonly, the disease is acquired by the grubs ingesting spores along with their food. Disintegration of diseased grubs, following death, serves to liberate spores to the soil. Although intact grubs may serve as sources of spores in the transmission of disease, dissemination is much more prompt when the spores are in direct contact with the soil.

Several experiments demonstrate the importance of heavy grub populations for a rapid spread of disease. A high inoculum potential also favors spread. A heavy grub population can compensate for a low inoculum potential and, conversely, a heavy inoculum potential can compensate for a low population in causing a resultant high incidence of milky disease.

The spread of disease was observed over periods of time among third instar grubs and among developing populations of grubs reared under different conditions. In several instances, an increasing inoculum did not result in a progressive increase in the incidence of disease. Instead, a period of increasing morbidity was followed by a decline. The reason for this is that the infection rate, at first, exceeds the mortality rate. The mortality rate then exceeds the infection rate. The slower infection rate may be due, in part, to an accumulation of the more resistant grubs.

As measured by bioassay methods, milky disease spores tend to remain more concentrated in the top two inches of soil, but there is some vertical spread of spores applied to the surface.

The role of *Bacillus popilliae* as a parasite is discussed, and comparisons are made between infection trends of the milky disease and characteristic epidemic curves.

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